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CONTENTS

No. 1, July 1, 1938

v

No. 2, August 1, 1938

No. 3, September 1, 1938

No. 4, October 1, 1938

CONTENTS

No. 6, DECEMBER 1, 1938

PAGE

HORMONE STUDIES WITH THE ULTRACENTRIFUGE

I. An Improved Air-Driven Vacuum Ultracentrifuge Suitable for Concentration Work in Biological Experiments

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While the ultracentrifuge to be described is essentially of the type originated by Beams and Pickels (1935),[1] some of the details of construction are new, and it is thought that they may be of value to those who wish to carry on work of this kind.

The distinguishing features of the instrument are described below.

1. A precession damping device, which prevents the centrifuge rotor from going into disturbing precessional motions at certain speeds during acceleration and deceleration. Although we do not claim originality for this idea, we do not know of any instance where it is being successfully used at present.

By preventing precessional motions, the damping device makes permissible the use of a longer section of shaft between the centrifuge rotor and lower bearing. This reduces the transmission of vibrations along the shaft from the centrifuge rotor to the lower bearings and driving mechanism, and greatly increases the life of the bearings.

2. Provision for adjustment of the positions of the driving mechanism parts, and for dismantling the driving mechanism without disturbing these adjustments.

3. A double compressed air turbine, which serves both as drive and brake.

4. Use of the flat disc type air bearing which permits smooth operation over a wide variation of air pressure.

The Driving Mechanism

Fig. 1 shows a cross section of the assembled driving mechanism, which is mounted on the steel cover plate (1) of the vacuum chamber. The driving

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[1] Beams, J. W., and Pickels, E. G., *Rev. Scient. Instr.*, 1935, 6, 299.

mechanism is made of brass.   Where other materials are used in its construction, they will be specified in the description.

The English system of measurement is used in preference to the metric system, since it is more adaptable to the stock parts and tools available in ordinary supply houses and machine shops.

The lower bearing consists of the two phosphor bronze bushings (2) and (2'), mounted in the ends of the core piece (3).   Through these bushings passes the spring steel shaft (4).   The core piece rests between two duprene rings (5), and is held in place in the lower bearing housing (6) by the lower bearing retainer (7).
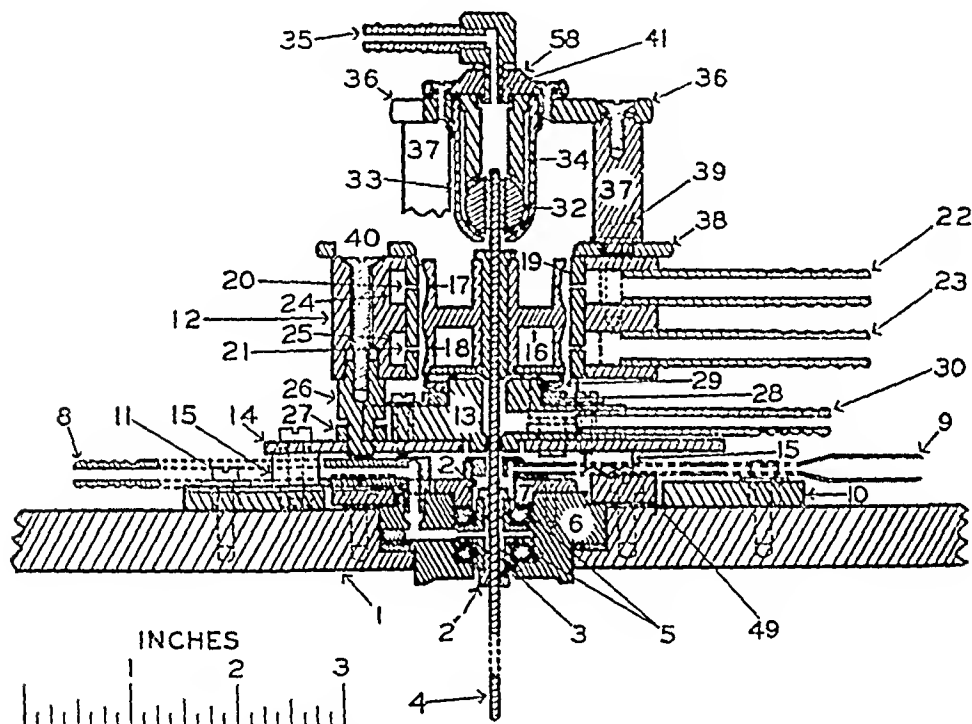
Fig. 1. The assembled driving mechanism.

Oil enters the housing through the oil inlet tube (8), and the oil escaping from the top of the bearing is led off through the oil drain (9).

The rest of the driving mechanism is mounted on the base ring (10), which is fastened to the cover plate by the six screws (11).   These screws pass through holes in the ring large enough to permit about 1/16 inch lateral adjustment.   The stator body (12), and the air bearings base (13), are carried by the base plate (14).   By placing thin shim washers on the shoulders of the three posts (15), the position of the plate is adjusted so that its plane is perpendicular to the direction given the shaft (4) by the set of the duprene rings (5).   If these rings are reasonably uniform, the shim washers should not have to be over 1/64 inch thick, and will not give the base plate enough tilt to be noticeable.

The turbine rotor (16) is made of duraluminum, and is mounted on the shaft (4). It has two identical sets of flutes (17) and (18), one of which runs clockwise and the other counter-clockwise. The air jet ring (19) of the stator has two identical sets of air jets (20) and (21), corresponding to the two sets of flutes on the rotor. The upper and lower sets of jets may be used interchangeably as drive and brake. In the present instrument the upper set is used to drive the rotor. Air is supplied to the jets through the inlet tubes (22) and (23), which connect with the annular channels (24) and (25).

The stator body (12) is mounted on the three steel studs (26), which are screwed into the base plate (14). These studs are adjustable in height and are locked in position by the nuts (27). The position of the base ring (10) is adjusted so that the stator and rotor are concentric when the rotor is in the position determined by the set of the lower bearing.

The entire weight of the rotating system is carried by a cushion of compressed air in the air bearing (13), (28), (29). Compressed air enters the air bearing base (13) through the inlet tube (30) and raises the turbine rotor (16) off the formica collar (29), the air escaping between the upper rim of the collar and the bottom of the rotor. During operation the rotor floats entirely free of the collar, the cushion of air maintaining a gap of a few thousandths of an inch between the rim of the collar and the under surface of the rotor.

The three screws fastening the air bearing base to the base plate (14) pass through holes large enough to permit about 1/16 inch lateral movement, thereby allowing the air bearing base to be adjusted so that the shaft passes concentrically through the hole (31) (see Fig. 4 A).

The upper bearing consists of a phosphor bronze bushing fitted centrally into the steel ball (32). This ball rests between duprene washers, and is held in place in the bottom of the housing (33) by the piece (34). Oil is admitted to the upper bearing through the oil inlet tube (35).

Three arms (36), resting on the posts (37), carry the upper bearing housing. These posts are mounted on the ring (38), which is fastened to the stator body (12) by the three screws (39). The screws pass through holes large enough to allow about 1/16 inch lateral movement of the ring, permitting the upper bearing to be centered with the shaft. Three large holes (40) are provided in the ring, so that the screws holding down the stator body may be removed without disturbing the adjustment of the ring.

Oil is supplied under pressure to the upper and lower bearings from a tank mounted on the vacuum chamber cover plate. This tank, which is filled with Cenco vacuum pump oil No. 93050, has a capacity of 280 cc. and holds enough oil for many runs. A glass gauge on the side indicates the oil level, and the oil is conducted to the bearing oil inlets (35) and (8) through 1/4 inch duprene tubing connected to outlets on the tank.

Since the pressure (9 to 10 pounds per square inch) required by the air bearing is also suitable for forcing oil to the upper and lower bearings, the top of the oil tank and the air bearing inlet are joined by a T connection so that they are both supplied by the same air hose.

The centrifuge rotor which hangs on the end of the shaft (4) is quite heavy, and it is essential that the shaft be held vertically during operation in order not to strain it.   To aid in proper orientation of the shaft, there is screwed to the vacuum chamber cover plate a steel framework carrying a small plumb bob.   The plumb bob hangs on a fine thread, and its point is $3\frac{1}{2}$ inches below the support to which the thread is fastened.   Mounted on the framework below the plumb bob, and facing upward, is a similar point whose position is adjustable, and which can be locked in place by a screw.

After the driving mechanism has been assembled and adjusted, the rotor and shaft are removed and the upper bearing is put back in place.   A thread is then passed through the lower and upper bearings and out of the oil inlet hole (41) in the upper bearing cap.   The lower end of the thread is weighted, and the vacuum chamber cover plate is oriented so that the thread hangs centrally through both the upper and lower bearings.   The lower point on the plumb bob framework is now adjusted so that it is directly under the plumb bob point, and is then locked in place.

The position of the centrifuge is checked before each run to see that the points are lined up, thus insuring vertical alignment of the shaft during operation.

Details of Construction of the Driving Mechanism

The Turbine Rotor and Stator

The duraluminum turbine rotor, with its two oppositely directed sets of flutes, is shown in Fig. 2 A.   It is carefully machined in order to be as accurately balanced as possible.   The flutes were milled to a depth of 3/64 inch with a special cutter, and there are 30 flutes in each set.   The rotor is hollowed out to reduce its weight, and the bottom is closed by a duraluminum disc (42), pressed into place and turned off.   It was found necessary to close the bottom of the rotor to get rid of vertical vibration of the rotating system, which occurred when the bottom of the rotor was left open.   The inner rim of the top part of the rotor is inclined slightly outward, as shown in Fig. 2 A, so that it will clear itself of oil while running.   A phosphor bronze core (43) which is pressed into place, permits the rotor to be soldered to the shaft (4).

The shaft (4) is made from 3/32 inch straightened music wire,[2] and is about $7\frac{3}{4}$ inches long.

The stator body and air jet ring are shown in Figs. 2 B and C.   The air jet ring (19) has an inside diameter of $1\frac{3}{8}$ inches, so that the turbine rotor has a total clearance of 1/16 inch.   Its two oppositely directed sets of air jets (20) and (21) consist of eight holes in each set.   The air jet holes were made with a No. 53 drill, and point so that their axes pass 3/32 inch from the inner surface of the ring, as shown in Fig. 2 C.

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[2] Obtained from the Wickwire Spencer Steel Company, New York City.

The inside of the stator body (*12*) is bored out to form a light press fit for the air jet ring, which is soldered into place. The air channels (*24*) and (*25*) are supplied through the inlets (*44*) and (*45*), which are threaded to fit standard 1/8 inch pipe nipples.

FIG. 2. The turbine rotor and stator.

The Lower Bearing

The phosphor bronze bushings (*2*) and (*2'*) (see Fig. 3 A) are first screwed and soldered into the core piece (*3*), and are then drilled and reamed to fit the shaft. A section of shaft, which has one end hardened and ground off diagonally, is used to ream the bushings to final size. The rim (*46*) of the core piece flange is grooved for oil, which has access to the bearings through four holes (*47*) drilled in the flange. Since the lower bearing bushing (*2'*) projects into the vacuum chamber, it is made a little longer than the upper one in order to keep oil from running through it too fast.

The lower bearing housing (*6*) (see Fig. 3 C) fits snugly into place in the vacuum

chamber cover plate, and its outer rim is slightly rounded to prevent binding. A vacuum tight seal is obtained by placing under the shoulder (48) a lead washer smeared with Cenco vacuum wax, and clamping the housing tightly into place by the steel ring (49), shown in Fig. 1.

The core piece well (50) has a diameter 0.013 inch greater than the core piece flange, in order to permit free cushioning action of the duprene rings (5), Fig. 3 B. Oil enters the core piece well through the opening (51), which is placed so as to be
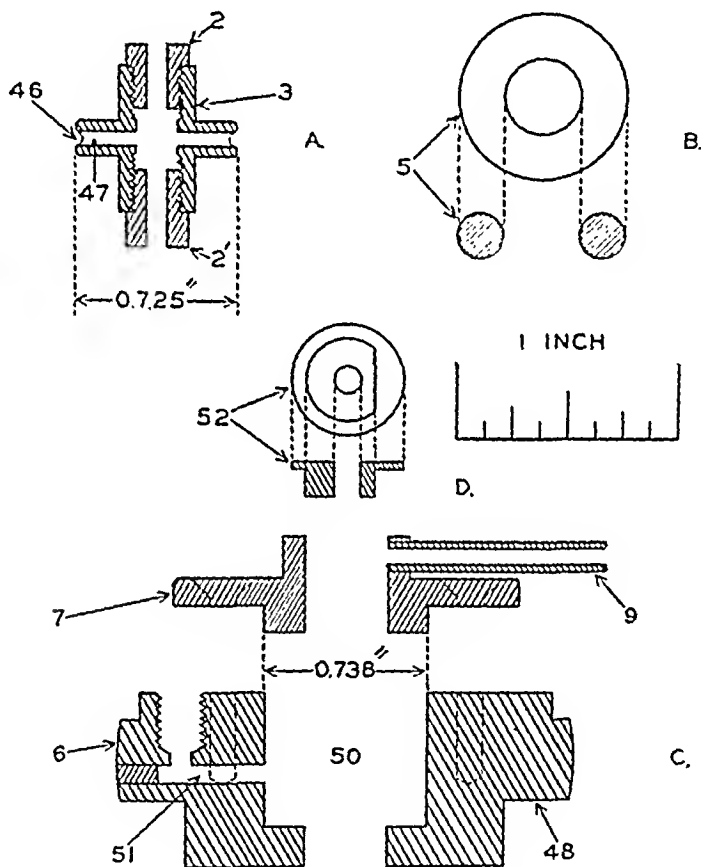
FIG. 3. The lower bearing and housing.

even with the core piece flange when the assembly is fastened in place by the lower bearing retainer (7), Fig. 3 D.

A small cap (52) fits into the top of the lower bearing retainer and serves to protect the bearings. One side of the cap is cut away so that it will not block the oil drain (9), which is made from a flattened piece of 1/4 inch tubing.

The core piece should be held in place in the well firmly, but not too rigidly. In order to permit the duprene rings to absorb small vibrations that might be present, they should be allowed a slight amount of "give."

The Air Bearing

The assembled air bearing, mounted on the base plate (*14*) and carrying the turbine rotor (*16*), is shown in Fig. 4 A. The hole (*31*) through which the shaft enters the bearing base (*13*) should be large enough to clear the shaft, but not large enough to allow the escape of too much air. About 0.020 inch clearance is left between the rotor and the neck of the air bearing base. It was found that if this space is much larger, the escaping air sets up an annoying whistle, which disappeared when the clearance was reduced to the dimensions given.
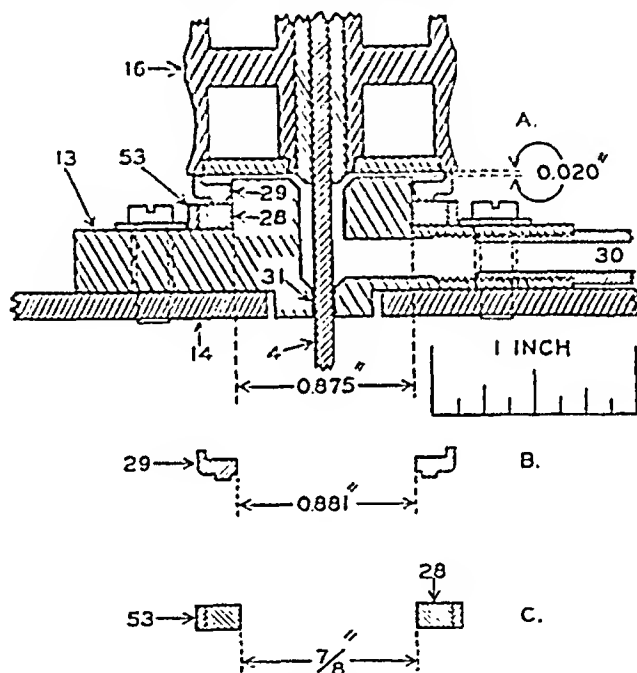
FIG. 4. The assembled air bearing.

The formica collar (*29*) (see Fig. 4 B) should be made as light as possible, but not too fragile. It rests loosely around the neck of the air bearing base, having about 0.006 inch clearance to prevent binding.

The duprene washer (*28*), Fig. 4 C, which carries the formica collar, was cut from a 1/8 inch thick duprene sheet. It is enclosed in a thin aluminum ring (*53*), which keeps it from expanding under the applied air pressure.

The Upper Bearing

Fig. 5 shows the upper bearing, assembled in its housing (*33*). The phosphor bronze bushing (*55*) is drilled and reamed to fit the shaft in the same manner as the

lower bearing bushings, and is then turned to size on an arbor and pressed into place in the steel ball (*32*).

The ball (*32*) is a steel ball bearing, which is annealed, bored to accommodate the bushing, and then rehardened and tempered. The ball rests firmly between a pair of 1/32 inch thick duprene washers (*57*), but is not clamped tightly enough to destroy their cushioning action.

By loosening the screws holding down the bearing cap (*58*) and inserting a short section of shaft into the bearing, the ball can be turned to align the bearing with the shaft.
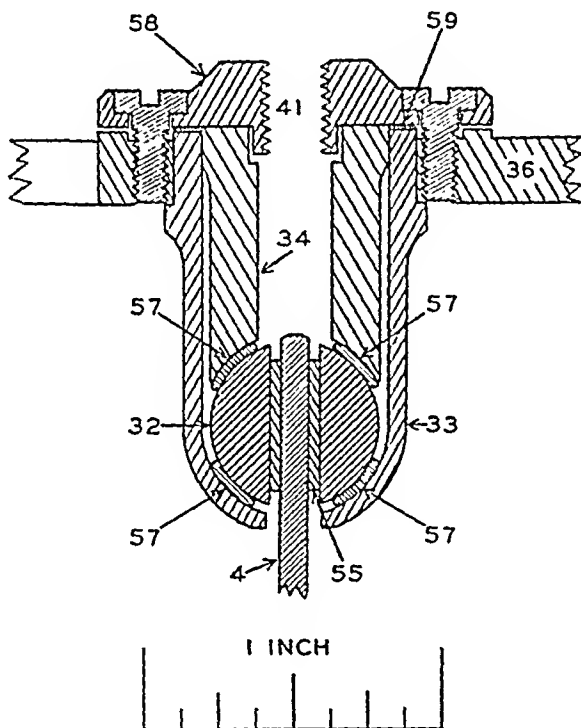
FIG. 5. The assembled upper bearing and housing.

A thin paper gasket (*59*) placed between the cap and the rim of the housing keeps oil from leaking out under the cap.

The Vacuum Chamber Assembly

The Centrifuge Rotor

The centrifuge rotor is shown in position in the vacuum chamber in Fig. 6. It is patterned closely after the one described by Bauer and Pickels (1936),[3] with the

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[3] Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, 64, 503.

exception that it is smaller and has fewer test tube holes. There is also a shoulder left at (60) making it possible for the rotor body to have a relatively wider mouth. The rotor body is 6⅛ inches in diameter, and the complete rotor weighs about 6.7 pounds.

The material used for the rotor body and cap (61), (62), is a duraluminum alloy having a very high ratio of tensile strength to density.[4] The rotor body (61) contains 10 test tube holes (63), inclined at 45° to the axis. These holes are bored to a diameter of 0.520 inch to accommodate the flexible test tubes.[5] The test tubes, as furnished, are about 4 inches long. They are cut down to 2½ inches for use in the rotor, so that 1/4 inch of the rim projects above the holes (63).
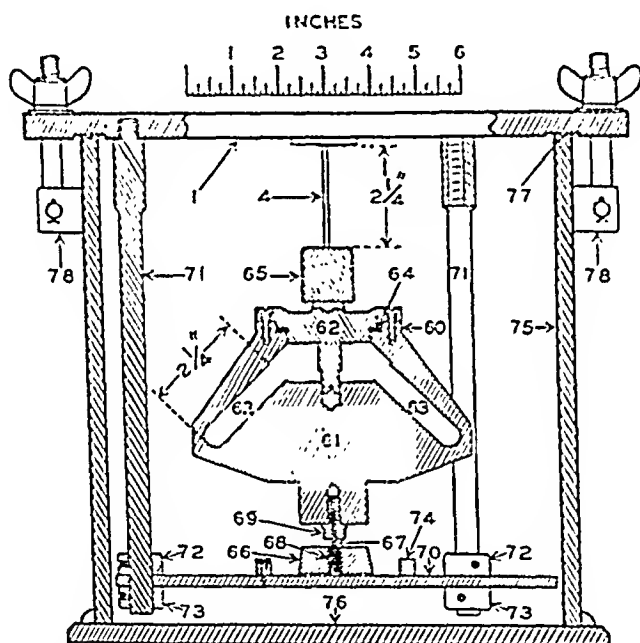
FIG. 6. The vacuum chamber, with the centrifuge rotor and precession damper.

During operation they can each conveniently hold 5.5 cc. of fluid, making the total capacity of the rotor about 55 cc.

A rubber washer (64), smeared with Cenco vacuum wax, forms a vacuum tight

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[4] This material is a special alloy known as 14 ST forged, and is manufactured by the Aluminum Company of America, Pittsburgh, Pennsylvania.

[5] Manufactured by the Lusteroid Container Company, South Orange, New Jersey. These test tubes are retailed by the International Equipment Company, Boston, Massachusetts.

seal for the rotor cap. The rotor is fastened to the end of the shaft (*4*) by a small chuck (*65*). (For a description of this chuck, see reference 3 on page 8.)

Unless the rotor is perfectly balanced, the shaft (*4*) will not coincide exactly with the principal axis of the rotor and will therefore be subjected to some vibration at the point where it enters the chuck (*65*). Even if the rotor is perfectly machined and the weight of each test tube with its contents is exactly the same, there will probably still remain a slight amount of unbalance because of variations in density of the metal. Hence the chuck of the rotor is placed about 2¼ inches below the lower bearing (see Fig. 6), so that the free section of spring steel shaft in between effectively protects the lower bearing from vibrations arising from slight unbalance of the rotor.

No perceptible vibrations are transmitted through the shaft to the bearings and driving mechanism, even though no attempt was made to balance the rotor after machining it, and though the test tubes and contents are not weighed to see that they match. In practice, the test tube contents are measured out with a 5 cc. Luer syringe.

The Precession Damper

At certain speeds a freely suspended rotor on the end of a long, flexible shaft is apt to go into pronounced precessional motions that are hard on the shaft and lower bearings. Such motions usually appear at relatively low speeds, during acceleration or deceleration of the rotor. In the centrifuge described they are eliminated by the damping device (*66*) to (*69*), Fig. 6. This device consists essentially of a duprene block (*66*), which is free to slide on the oiled steel plate (*70*). A short steel pin (*67*) on the bottom of the rotor enters a bushing (*68*) in the duprene block, so that if precessional motions are set up, the block is dragged around with the rotor. The friction between the duprene block (*66*) and the steel plate (*70*) is sufficient to damp these precessions so that they have no chance to build up. With the vacuum chamber cover plate suspended on a wooden frame so that the rotor could be observed while spinning, it was found to return quickly and smoothly to its normal position after being intentionally pushed aside.

The steel pin (*67*) is made from a piece of the 3/32 inch music wire used for the drive shaft (*4*), and is soldered into the steel plug (*69*). The bushing (*68*) is made from ordinary writing paper, rolled tightly on the shank of a 7/64 inch drill and pushed into the hole in the duprene block (*66*). This allows 1/64 inch clearance for the steel pin (*67*), so that after the rotor settles down, the pin comes to rest in the center of the bushing. The same paper bushing has been used for many runs without showing signs of wear, indicating that after the rotor has passed through its critical speeds the pin no longer touches the bushing.

Care must be taken to wind the paper bushing in the right direction, so that it will not roll up on the pin and seize it.

The duprene block (*66*) is made from a one hole No. 8 duprene stopper, cut down to 5/8 inch in height. The bottom of the block is covered with light card-

board, which has corrugations cut in it to prevent oil from sealing it to the plate (70). The cardboard is fastened to the bottom of the block with shellac.

The plate (70), which is made of 1/4 inch steel, is held in place on the three steel rods (71) by the collars (72), (73). These collars are adjusted so that the plate is horizontal when the plumb bob on the vacuum chamber cover plate hangs true, and so that the surface of the plate clears the end of the steel pin (67) by about 1/16 inch. The plate is removed by taking off the lower set of collars (73), while the upper set (72) preserves the adjustment. The wooden frame mentioned above facilitates removing and replacing the plate, by holding the ends of the rods (71) free of the table.

Three 1 inch holes (not shown in Fig. 6) are drilled in the plate (70), to insure good evacuation at low pressures.

To keep the rotor from swinging too far should the assembly be accidentally tipped during handling, there is screwed to the plate a formica guard ring (74). The bushing, pin, and section of plate inside the guard ring are copiously oiled with vacuum pump oil before each run.

The Vacuum Chamber

The vacuum chamber (see Fig. 6) is made from a section of 10 inch steel pipe (75), 3/8 inch thick, brazed to a 7/16 inch steel plate (76). The rim of the chamber has a groove (77) turned in it to fit a tongue in the cover plate (1), and the cover plate and rim of the chamber are carefully ground together to form a good fit. A coating of Cenco vacuum wax smeared on the chamber rim serves to make the joint vacuum tight.

To press the cover plate firmly into place before evacuating, the chamber is provided with six swivel bolts (78), which are swung up into slots in the projecting rim of the cover plate and tightened down with wing nuts.

A Cenco megavac pump, connected by a short length of 5/8 inch I.D. rubber vacuum tubing to a 1/2 inch pipe nipple in the chamber wall, is used to evacuate the chamber. Two 1/4 inch nipples provide connections for a McLeod gauge to measure the pressure in the vacuum chamber, and for a stopcock to let air back into it.

The weld at the bottom of the chamber was coated with piccin cement, and the chamber and pipe fittings were given two coats of glyptal paint.

To guard against danger from possible explosion of the rotor, the centrifuge is operated inside a heavy barricade 43 inches high, constructed of wood and sand. The front, facing the operator, is a double wall of 5 inch beams with 6 inches of sand between, forming a barrier 16 inches thick. One side is mounted on rollers to give access to the interior. Plyboard facing and aluminum corner moulding serve to give the barricade a finished appearance.

Inside the enclosure is a small wooden table, 10 inches high and covered with 1/2 inch thick sponge rubber, which supports the centrifuge. A pair of suitably placed mirrors enables the operator to see the top of the centrifuge over the front

of the barricade.   The top of the enclosure is covered by a removable glass plate, which serves to keep out dust and to confine the noise produced by the turbine during acceleration or braking with 20 to 30 pounds of air pressure.
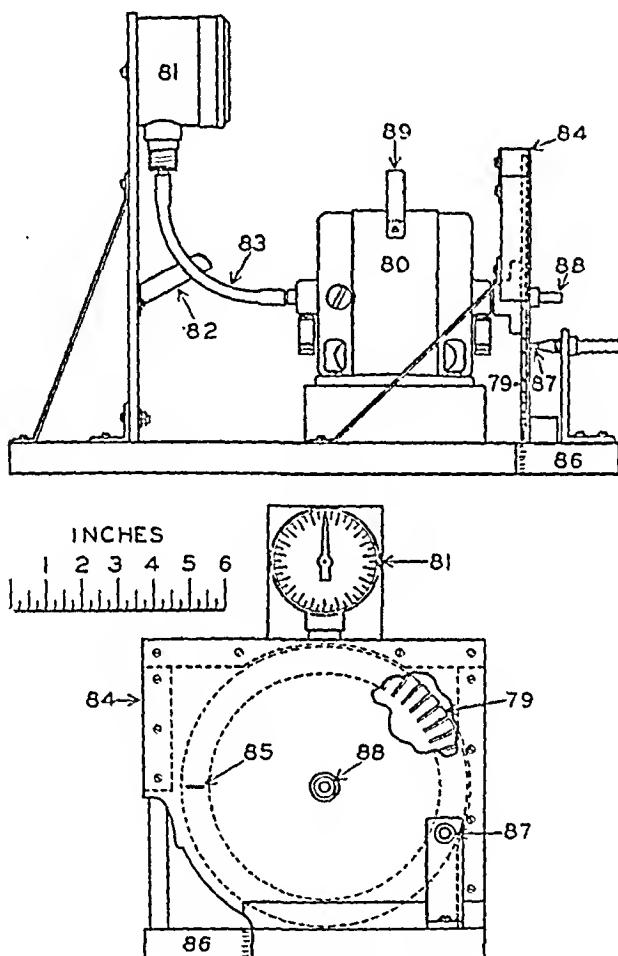
FIG. 7. The stroboscope.

Speed Measurements

The speed of the centrifuge is measured with the portable disc stroboscope shown in Fig. 7.   The stroboscope disc (79), mounted on the shaft of the small D.C. motor (80), is made of brass and is carefully balanced.   It is 8 inches in diameter and 1/8 inch thick, and has 60 radial slots cut in its rim.   Each slot is 1/16 inch wide and 3/4 inch deep.   The motor is 1/20 h.p., shunt wound, with a normal no-load speed of 1800 R.P.M.   By means of a potentiometer arrangement its speed can be varied from zero up to several thousand R.P.M.

At (*81*) is a small centrifugal type tachometer,[6] which is connected to the rear of the motor shaft by 1/4 inch rubber tubing, and which has a range of 200 to 2400 R.P.M. The piece (*82*) steadies the driving tube (*83*) by bearing lightly against it.

In front of the stroboscope disc there is a steel shield (*84*). This shield has a slot cut in it at (*85*), 1/2 inch long and 3/64 inch wide, against which the observer places his eye to view the turbine rotor through the slots in the revolving disc. The shield is painted black to eliminate reflections, and the shield and part of the base at (*86*) are cut away to accommodate the observer's face.

At (*87*) is mounted an air jet with a rectangular nozzle, placed opposite the slots in the stroboscope disc. The nozzle of the jet passes through a hole in the shield and terminates about 1/16 inch from the disc, with its wider axis parallel to the slots passing before it. The jet is provided with an air hose nipple, and together with the disc forms a siren whose pitch can be controlled by the speed of the motor. Since there are 60 slots in the disc and since the tachometer is calibrated in revolutions per minute, the pitch of the note in vibrations per second produced by the siren is indicated directly by the tachometer dial.

This arrangement provides a second means of checking the speed of the centrifuge, besides the customary method of observing the turbine rotor through the slotted disc. During operation the centrifuge emits a clear tone whose frequency is equal to the speed of the turbine rotor. Hence, by matching the tone of the siren with the tone of the centrifuge, the observer can obtain the speed of the centrifuge in revolutions per second directly from the tachometer dial.

The audible method of speed checking was found to be as convenient and accurate as the visual method, with the added advantage of letting the operator know at all times in which direction to change the speed of the stroboscope when seeking synchronism. When using the visual method alone, it is hard to tell whether the stroboscope is above or below synchronous speed until synchronism is nearly reached.

The tachometer may be tested for accuracy from time to time by attaching a revolution counter to the stud (*88*), which projects from the motor shaft through the front of the shield.

A handle (*89*), screwed to the motor frame, enables the stroboscope to be easily carried around.

Operation

After the flexible test tubes have been equally filled by means of a pipette they are put into the rotor and the rotor cap is screwed in place, the cap being rendered air tight by the rubber washer (*64*) (see Fig. 6). It is not necessary to fill the tubes to capacity, but reasonable care should be taken to have the same volume of fluid in each.

The rotor is then placed on the shaft and the chuck (*65*) is screwed down as

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[6] Manufactured by the Jones Motrola Company, Brooklyn, New York.

tightly as possible by hand.   It was found unnecessary to use a wrench on the chuck, since it has shown no tendency to work loose.   While tightening the chuck it is well to have the air supply to the air bearing turned on, in order to avoid twisting the shaft.   A small ring marked on the shaft aids in placing the rotor so that the pin (*67*) will clear the plate (*70*) properly.

When the rotor is in position, the damping block (*66*) is slipped on the pin (*67*) and held while the plate (*70*) is raised into place against the upper set of collars (*72*).   The plate is then fastened by the lower set of collars (*73*).

The rim of the vacuum chamber is next well coated with Cenco vacuum wax, care being taken to see that the rim of the chamber and the ground surface on the lower side of the cover plate are free from dirt.   Then the cover plate with the rotor, driving mechanism, etc., is lowered into place with the aid of a small derrick mounted on the front of the barricade, and is firmly seated on the vacuum chamber by means of the swivel bolts (*78*).

After the vacuum chamber is pumped down, the air bearing pressure is set between 9 and 10 pounds per square inch, and the machine is started.   Care should be taken before starting to see that the plumb bob on the vacuum chamber cover plate hangs true, in order to insure vertical alignment of the drive shaft and horizontal alignment of the plate (*70*).

DISCUSSION

The maximum operating speed used for this instrument is 850 R.P.S., which gives a centrifugal field of over 100,000 times gravity at the top of the fluid column in the test tubes and a field of over 200,000 times gravity at the bottom.   Higher speeds than this have not been attempted because of the danger of explosion.[7]

With a pressure of 30 pounds per square inch on the driving turbine, the rotor attains its full speed of 850 R.P.S. in about 35 minutes. About 9 pounds per square inch is sufficient to maintain this speed after it is reached.   15 to 20 pounds per square inch on the braking turbine brings the rotor to a stop from full speed in about 30 minutes. Several hours are required for the rotor to coast to a stop from full speed if the brake is not used.

[7] For some months after the machine was put in operation, it was not run at speeds higher than 650 R.P.S.   On the basis of experiments by Wyckoff and Lagsdin (*Rev. Scient. Instr.*, 1937, 8, 427), testing the strength of similar rotors, it was decided to increase the maximum operating speed to 850 R.P.S.   Micrometer calipers were used to keep a check on the dimensions of the rotor.   Although many runs of from 2 to 6 hours duration have since been made at this speed, careful measurements of the diameter of the rotor at its rim and mouth have shown no detectable stretching.

The air bearing does not require a critical adjustment of air pressure. Although the pressure is usually kept between 9 and 10 pounds per square inch, the air bearing has been found to operate satisfactorily anywhere in the range between 8 and 12 pounds per square inch. The pressure has been varied between these limits while the centrifuge was in operation without causing trouble.

During acceleration and deceleration one or two small vibration periods appear, but they are not pronounced enough to cause trouble, and the centrifuge passes through them without difficulty. In the usual operating speed range of 600 to 850 R.P.S. the machine is very smooth and quiet, emitting only a faint clear note. The plate glass cover must be removed from the top of the barricade after the centrifuge attains full speed in order that the operator may hear the tone and match it with the siren.

Many 6 hour runs have been made without developing any bearing trouble. The small amount of oil running into the vacuum chamber through the lower bearing has not been found sufficient to make necessary a special collecting device. A small amount also escapes from the upper bearing, but this likewise causes no particular inconvenience.

During operation the pump maintains the pressure in the vacuum chamber at 3 or 4×10^{-3} mm. of mercury, as indicated by the McLeod gauge. No vapor trap is used in the system. Upon completing a run, the centrifuge rotor is slightly warm to the touch immediately after removing it from the vacuum chamber, showing that even at such low densities there is enough friction with the residual gas to cause some rise in its temperature. Good sedimentation results have been obtained under these vacuum conditions, however, and it is doubtful that sufficient temperature gradients are set up in the rotor to cause appreciable stirring of its contents.

The great centrifugal forces set up in the centrifuge rotor cause the rims of the test tubes to fold over and close the mouths of the tubes. Fractioning of the contents is greatly facilitated by inserting a hypodermic needle through the folded rims and drawing off the upper fractions through it. In order to minimize stirring, this may be done before the test tubes are removed from the rotor. The last fraction is best removed after the test tubes have been taken out. A pair of

hemostats have been found very convenient for pulling the test tubes out of the rotor.

SUMMARY

1. An ultracentrifuge is described in which the rotor is driven by a compressed air turbine, and is spun in an evacuated chamber to minimize friction and heating. The rotating parts are supported by a cushion of air in an air bearing.

2. The centrifuge rotor holds 10 test tubes inclined at 45° to the axis, and has a capacity of 55 cc. It is operated at a maximum speed of 51,000 R.P.M., which develops at the top of the fluid column in the test tubes a centrifugal field of over 100,000 times gravity, and at the bottom of the fluid column a field of over 200,000 times gravity.

3. By means of a reverse turbine, the rotor can be brought to a stop from full speed in a relatively short time.

4. A precession damping device is described, which effectively damps the precession and wobbling of the rotor that usually occurs at certain speeds in machines of this type.

5. A relatively long section of shaft is used between the centrifuge rotor and lower bearings. This prevents vibrations from being appreciably transmitted through the shaft to the lower bearings and driving mechanism, and results in a negligible wear on the bearings.

6. The driving mechanism is designed so that the positions of its parts are adjustable, and so that the driving mechanism may be dismantled without disturbing these adjustments.

EFFECT OF REPEATED SUPERINFECTION UPON THE POTENCY OF IMMUNE SERUM OF MONKEYS HARBORING CHRONIC INFECTIONS OF PLASMODIUM KNOWLESI

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In a previous communication (1) it was reported that pooled serum obtained from monkeys with chronic infection of *Plasmodium knowlesi* contained protective antibodies as evidenced by the ability to protect normal animals from death due to infection produced by the injection of homologous parasites. The fact that large amounts of immune serum are necessary to produce an inhibitory effect upon the course of the experimental disease was taken as an indication that the antibodies were present in the serum of such animals in a rather low concentration. In addition, it was observed that the potency of the immune serum decreased progressively after repeated bleedings of the same donor animals. The purpose of this paper is to report attempts made to increase the potency of the immune serum in monkeys with chronic infections resulting from single or multiple injections of massive doses of homologous parasites.

Materials and Methods

Fifteen *rhesus* monkeys whose original acute attacks of *P. knowlesi* malaria had assumed a chronic form for a period of at least 8 months were used as source animals for the immune serum. These animals had been bled repeatedly for immune serum in earlier experiments (1). It was noticed, however, that after each bleeding there was an apparent diminution in the protective property of their serum. After about a month's rest from previous bleeding these animals were given a series of intravenous injections of massive doses of parasites at various time intervals. The parasites used for superinfection were obtained from a number of monkeys which were suffering from acute attacks of *P. knowlesi* infection, and they were bled shortly before death when their parasite count in the blood

17

was at its maximum density. The immune serum obtained from the superinfected animals was either pooled or kept separately as required for the particular experiment. For protection tests normal *rhesus* monkeys of approximately equal age and weight were used. The number of parasites used for the inoculum in each protection test was approximately 1,500,000, and the amount of infected blood necessary to contain this number was computed beforehand by making a simultaneous red blood cell and parasite count upon the source monkey. In the first few protective tests immune serum and parasites were injected separately into the abdominal cavity of the monkeys. In one experiment the two components were mixed and incubated at 37°C. for 30 minutes before injection. Parasite counts were made daily on all animals under observation by means of thin blood smears.

EXPERIMENTAL

Experiment 1.—The *rhesus* monkeys harboring chronic infections of varying durations were divided into 2 groups. Group 1 consisted of 8 monkeys, Nos. 1 to 8 inclusive, and group 2 of 7, Nos. 9 to 15 inclusive. The animals in group 1 were given a course of hyperimmunization which consisted of 7 injections of massive doses of parasites. These injections were given intravenously at approximately 7 day intervals, and the doses varied from 5 to 7 billion parasites. The monkeys in group 2 were allowed to continue their chronic course without intervention and were intended to serve as controls. 6 days after the last injection of parasites into group 1, the animals of both groups were bled and their serum collected into 2 respective pools. The serum collected from monkeys in group 1 was designated as pool 1, and that from group 2, pool 2. The following comparative protection experiment was carried out with these 2 pools.

As shown in Table I and Charts 1 and 2, 10 normal *rhesus* monkeys, Nos. 33 to 42 inclusive, were each inoculated with 1,500,000 *P. knowlesi* parasites. Immediately following the injection of the parasites and daily thereafter, 3 of the monkeys, Nos. 33, 34, and 35, were given intraperitoneally hyperimmune serum of pool 1 in amounts of 10 cc., 5 cc., and 2.5 cc. respectively, while a fourth monkey, No. 36, received 1 cc. of the same serum at similar intervals intravenously. Another group of 4 monkeys, Nos. 37, 38, 39, and 40, were each given serum of pool 2 in corresponding amounts and at the same intervals as those which received pool 1. Two additional control monkeys, Nos. 41 and 42, were given no serum.

As seen from Table I, the use of the immune serum in pool 1, which came from the superinfected group of monkeys, was followed by a very marked protective action. Monkey 33, which received 10 cc. of this serum daily for 10 days, never showed any detectable parasites in the peripheral blood. As a further proof for the protective action of this serum, blood from this monkey was subinoculated into normal

monkeys on six separate occasions at approximately weekly intervals during and following the protection test, and none of these developed malaria. Monkey 34 received 5 cc. of serum daily for 10 days, and parasites were first detected in its blood smear only on the 20th day after the original inoculation and reached a peak of 13 per 10,000 red blood cells on the 28th day. Monkey 35, which received 2.5 cc. of the same serum for 10 days, first showed parasites on the 13th day following inoculation; they reached a peak of 55 per 10,000 red blood cells on the 19th day, and then subsided into a chronic infection. Monkey

TABLE I

Experiment 1

Ten normal monkeys each inoculated with 1,500,000 parasites and immune serum of pools 1 and 2 given in doses and at intervals as follows:

| Monkey No. | Injection of immune serum | | Results |
	Pool	Dose and route	
33	1	10 cc. i. p. daily for 10 days	Survived
34	1	5 cc. i. p. daily for 10 days	Survived
35	1	2.5 cc. i. p. daily for 10 days	Survived
36	1	1 cc. i. v. daily for 10 days	Died on 10th day
37	2	10 cc. i. p. daily for 10 days	Died on 14th day
38	2	5 cc. i. p. daily for 10 days	Died on 12th day
39	2	2.5 cc. i. p. daily for 9 days	Died on 10th day
40	2	1 cc. i. v. daily for 7 days	Died on 8th day
41		Control, no serum	Died on 7th day
42		Control, no serum	Died on 9th day

i.p., intraperitoneally.
i. v., intravenously.

36, which received only 1 cc. of serum intravenously for 10 consecutive days, died on the 10th day.

While serum of pool 1 showed definite protective properties, that of pool 2 showed only a slight inhibitory effect upon the course of the experimental disease in monkeys in which it was tested. As shown in Table I, monkey 37, which received the greatest amount of this serum, died on the 14th day following inoculation, and monkey 38, which had been given 5 cc. of serum for 10 days, died on the 12th day. Monkeys 39 and 40, which received 2.5 cc. and 1 cc. respectively, died

Days following infection with *Plasmodium knowlesi*
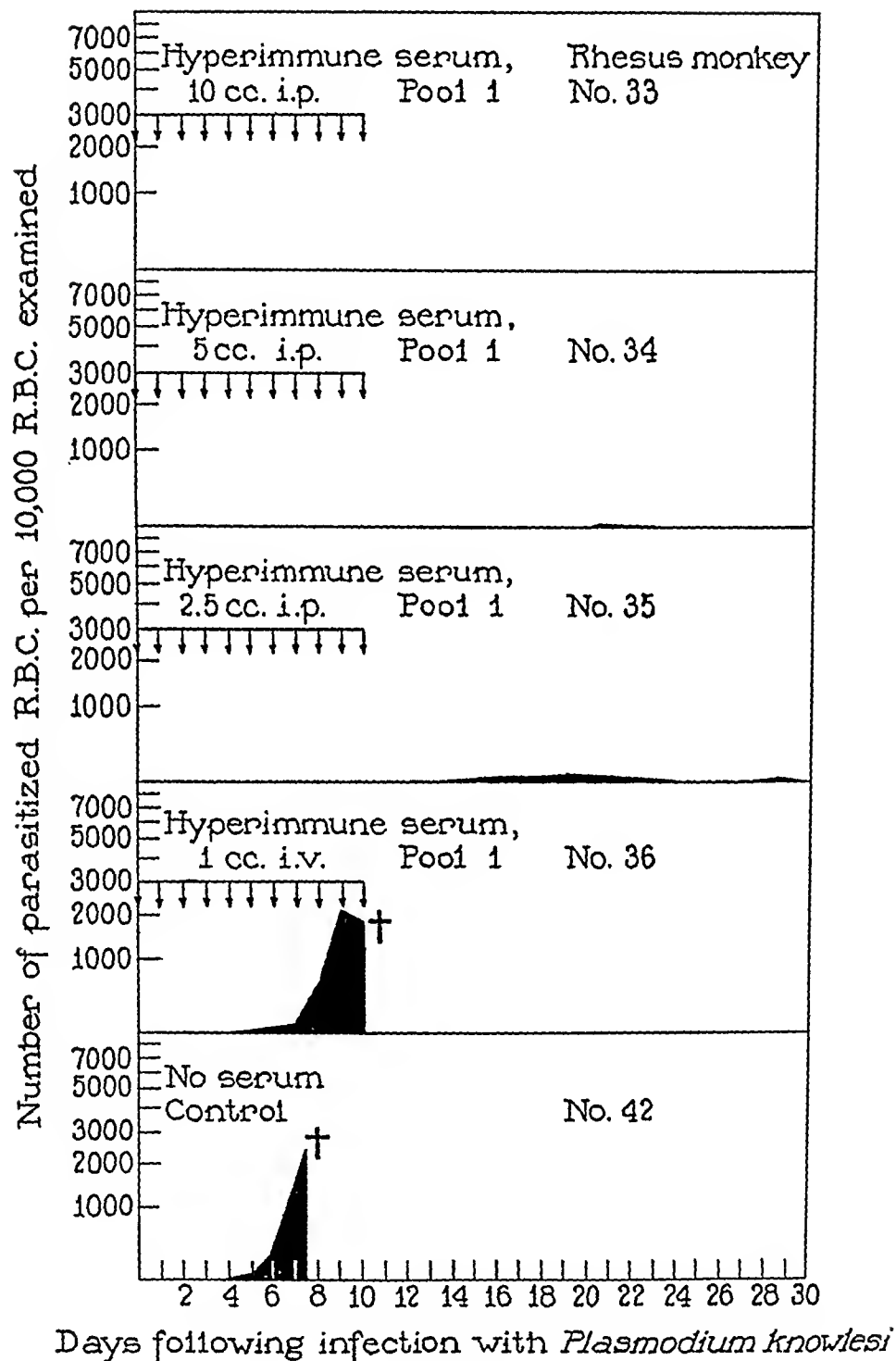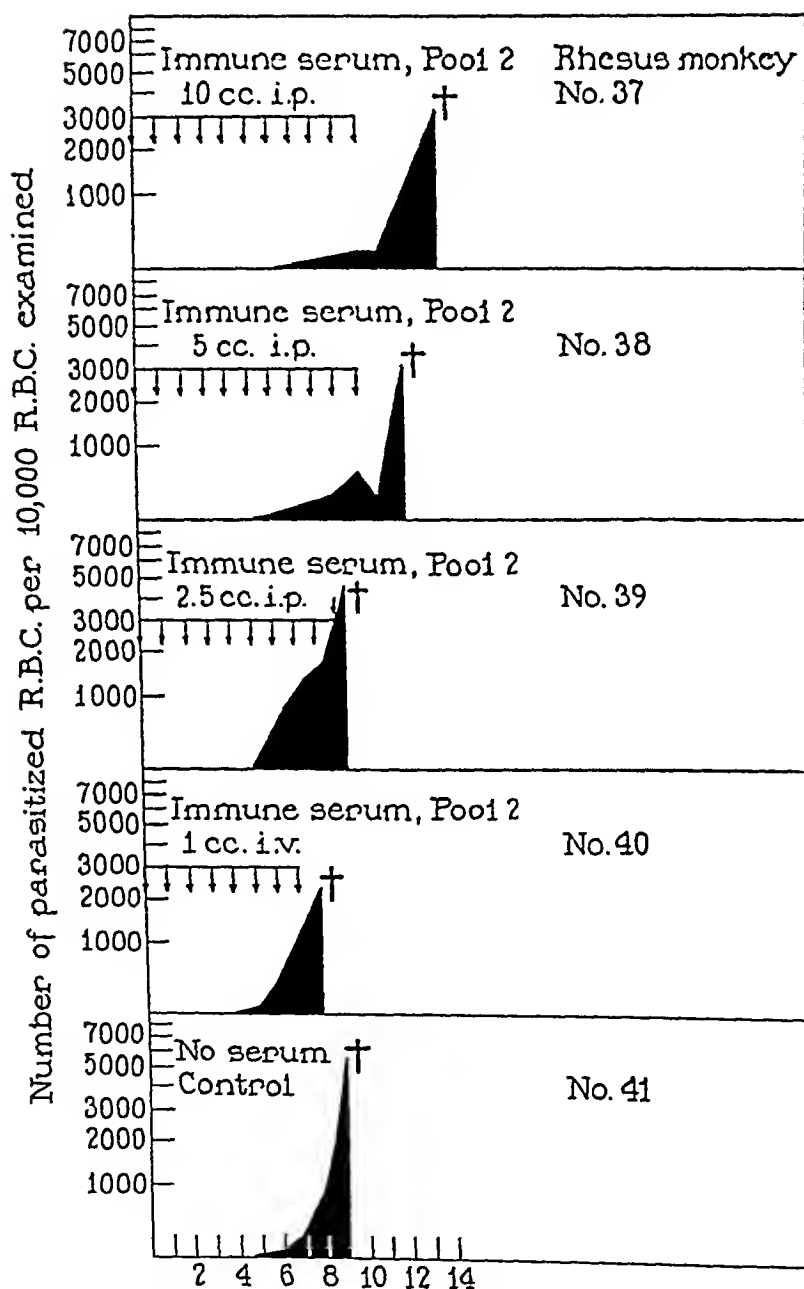
CHART 1

Days following infection with *Plasmodium knowlesi*

CHART 2

practically at the same time as the control monkeys, Nos. 41 and 42, which received no serum.

The results of this experiment indicate that the potency of the serum obtained from monkeys with chronic infections can be markedly increased by the repeated injections of large numbers of parasites.

Experiment 2.—The experiment above was repeated as follows: 4 monkeys of group 2, which harbored chronic infection and served as the source of serum for pool 2 used in the preceding experiment, were subjected to a prolonged course of hyperimmunization. At 6 day intervals they were given a total of 9 injections of parasites in doses varying from 2 to 13 billion. The total time required for this course of hyperimmunization was 2 months. On the 6th day after the last

TABLE II

Experiment 2

Five normal monkeys each inoculated with 1,213,000 parasites and immune serum of pool 3 given in doses and at intervals as follows:

Monkey No.	Injection of immune serum, dose, and route	Results
43	5 cc. i. p. daily for 10 days	Died on 12th day
44	2.5 cc. i. p. daily for 10 days	Died on 12th day
45	1 cc. i. p. daily for 10 days	Died on 12th day
46	1 cc. i. v. daily for 10 days	Died on 11th day
47	Control, no serum given	Died on 9th day

injection they were bled and the serum pooled, which was designated as pool 3. A protection test was set up with this pool as follows:

Five normal monkeys, Nos. 43 to 47 inclusive, were each inoculated intravenously with 1,312,000 parasites. Immediately after the injection of parasites and daily thereafter for a period of 10 days, monkeys 43, 44, and 45 were given 5 cc., 2.5 cc., and 1 cc. respectively, of serum from pool 3 intraperitoneally, and monkey 46 was given 1 cc. of same serum and at similar intervals intravenously. Monkey 47 received no serum and served as control. The results of this experiment are shown in Table II. It will be noted that this pool of serum showed practically no protective property as there was only a slight delay in the death of the animals which received daily doses of immune serum as compared with the control animal. Because of the inadequate amount of serum available, 10 cc. doses were not used in this experiment.

Experiment 3.—Seven monkeys with chronic infection, Nos. 2 to 8 inclusive, which in Experiment 1 had been included in group 1 and given a series of injections of massive doses of parasites, were now subjected to a second course of hyper-

immunization. Following a rest period of 1 month after the first course of hyperimmunization and bleeding, these monkeys were given 9 additional injections at 6 day intervals in doses varying from 2 to 13 billion parasites. On the 6th day after the last injection they were bled and the protective property of the serum of each tested separately as follows:

Eight normal monkeys, Nos. 48 to 55 inclusive, were each inoculated with 1,350,000 parasites. Seven of these were given serum from an equal number of hyperimmunized monkeys, while the eighth animal served as control. As shown in Table III, the serum of each hyperimmunized monkey was tested separately in a single animal; it was given intraperitoneally in 1 or 2 cc. amounts, beginning immediately after the injection of the parasites, and daily thereafter for 10 days, or until the death of the test animal.

TABLE III

Experiment 3

Eight normal monkeys each inoculated with 1,350,000 parasites. Immune serum from individual monkeys given intraperitoneally in doses and at intervals as follows:

Monkey No.	Source and dose of immune serum injected	Results
48	Serum from monkey 8 given 2 cc. daily for 10 days	Survived
49	Serum from monkey 4 given 2 cc. daily for 10 days	Died on 11th day
50	Serum from monkey 5 given 2 cc. daily for 9 days	Died on 9th day
51	Serum from monkey 6 given 2 cc. daily for 9 days	Died on 9th day
52	Serum from monkey 7 given 2 cc. daily for 8 days	Died on 8th day
53	Serum from monkey 3 given 1 cc. daily for 8 days	Died on 8th day
54	Serum from monkey 2 given 1 cc. daily for 8 days	Died on 8th day
55	Control, no serum given	Died on 8th day

The results are summarized in Table III. It will be noted that with a single exception all test monkeys died of malaria, showing that the serum of the hyperimmunized monkeys had no inhibitory effect upon the experimental disease. One animal, monkey 48 which received serum from hyperimmunized monkey 8, suffered a mild attack of malaria and survived. This monkey died a month later of generalized tuberculosis. The negative results of this experiment indicated that the monkeys had been excessively superinfected.

Experiment 4.—The results above indicate that immune serum of relatively high potency was obtained from monkeys after a series of 7 injections of massive doses of parasites in Experiment 1. On the other hand, the serum of the same animals, after a more intensive course of hyperimmunization consisting of 9

SUPERINFECTION IN MALARIA

injections given at similar intervals and in somewhat larger doses, had practically no protective property. It was now considered of interest to determine the effect of a single injection of parasites upon the antibody content of the serum of the same animals. Accordingly, a group of 10 monkeys harboring chronic infection were chosen as the source of immune serum. Seven of these, Nos. 2 to 8 inclusive, had already been subjected to two courses of hyperimmunization as described in Experiments 1 and 5, while three, Nos. 9, 12, and 14, had received a single course of 9 injections in Experiment 2. These 10 animals were allowed a rest of 1 month after their last hyperimmunization and bleeding and were then

TABLE IV

Experiment 4

Eleven normal monkeys each inoculated with 1,500,000 parasites. Immune serum from individual monkeys given intraperitoneally in doses and at intervals as follows:

Monkey No.	Source and dose of immune serum injected	Results
56	Serum from monkey 14 given 2 cc. daily for 10 days	Survived
57	Serum from monkey 4 given 2 cc. daily for 10 days	Died on 16th day
58	Serum from monkey 2 given 2 cc. daily for 10 days	Survived
59	Serum from monkey 5 given 2 cc. daily for 10 days	Survived
60	Serum from monkey 12 given 2 cc. daily for 10 days	Survived
61	Serum from monkey 8 given 2 cc. daily for 10 days	Survived
62	Serum from monkey 3 given 2 cc. daily for 10 days	Survived
63	Serum from monkey 6 given 2 cc. daily for 8 days	Died on 8th day
64	Serum from monkey 9 given 2 cc. daily for 10 days	Survived
65	Serum from monkey 7 given 2 cc. daily for 10 days	Survived
66	Control, no serum given	Died on 10th day

given a single injection of 7 billion parasites. One week later all were bled and the sera tested separately for protective antibodies as follows:

To 2 cc. of serum from each hyperimmune monkey were added 1,500,000 parasites and the mixtures incubated for 30 minutes at 37°C., after which each mixture was injected intraperitoneally into a separate normal monkey, while a control monkey received the same number of parasites incubated with 2 cc. of normal monkey serum. As shown in Table IV, the 10 test animals, Nos. 56 to 65 inclusive, each received 2 cc. of serum from a separate hyperimmune monkey daily for a period of 10 days following the injection of parasites. This also includes the amount mixed and incubated with the parasites at the time of inoculation. No subsequent injections of normal serum were given to the control monkey, No. 66.

The results are summarized in Table IV, and it will be seen that only 2 of the test animals died while 8 survived. The serum of mon-

keys 4 and 6 exhibited hardly any protective properties, although it must be pointed out that both of these monkeys suffered a relapse with a considerable number of parasites in the circulating blood at the time they were bled. In a later experiment the residue of serum from monkeys 3 and 12 was tested in a similar manner and against a similar number of parasites, except that the parasites and serum were injected separately. The test animal survived, indicating that the incubation of parasites and serum together had no influence upon the results of the above experiment.

DISCUSSION

A fundamental point in the study of immunity in malaria is the response of an animal with a chronic infection to superinfection. In the literature in this field are numerous reports which show that the superinfection of any animal with a chronic malaria infection by the injection of the homologous parasites results in a very rapid removal of the organisms from the blood stream (2–4). Information regarding the humoral immunity response to the injection of massive doses of homologous parasites is totally lacking. However, with numerous other infectious diseases it has been shown that the antibody titer of an animal can be elevated by hyperimmunization.

The results obtained in this experimental study indicate that the protective properties of immune serum of monkeys with chronic infections could either be increased or decreased by superinfection. An analysis of the outcome of the individual experiments deserves certain comment. In Experiment 1 it was noted, in the two comparable groups of immune monkeys, that those hyperimmunized had a much more potent protective serum than those which had been allowed to rest without superinfection. Of special interest was monkey 33 (Table I) in this experiment which was completely resistant to infection after receiving serum from the superinfected group. 6 weeks after the original inoculation this monkey, No. 33, was re-inoculated with exactly the same number of parasites as previously used. The second inoculation resulted in infection and death on the 19th day, which suggested that the animal had acquired no active immunity from the original inoculation but had retained enough passively acquired immunity to prolong the time of death.

The results of Experiment 2 and Experiment 3 show that after 9

superinfections the pooled sera of the four monkeys superinfected for the first time were no more potent than had been observed in Experiment 1 and that the sera from the 7 monkeys receiving the second course of superinfection and tested separately protected in only one instance. The survival of monkey 48 in this experiment cannot be attributed solely to the action by immune serum as it died of generalized tuberculosis 1 month after inoculation. It has been repeatedly observed that tuberculosis has an inhibitory effect on the course of *P. knowlesi* infection in monkeys.

The negative results of this experiment were unexpected. In view of the fact that the serum of the monkeys with chronic infection after the first course of hyperimmunization had shown a relatively high protective property, it had been expected that after the second course this property would be as high or even considerably higher. The only explanation that occurred to us as plausible for the failure was that repeated flooding of the animals with parasites apparently had exhausted most of the specific antibodies. This view is supported by the fact that, after the last injection of parasites and at the time of bleeding, most of the animals had a parasitic relapse, an occurrence which was absent during the course of hyperimmunization.

Experiment 4 showed that after a single superinfection all but 2 animals of those which had been used throughout as a source of immune serum showed protective antibodies. The blood of these 2 animals showed parasites at the time they were bled for the protection test, while the remaining 8 showed no parasites in their peripheral blood following the single superinfection.

We feel that the results of the present experimental work indicate that it is possible to influence the immune state of a monkey with chronic *P. knowlesi* infection by superinfection. The exact degree of alteration is difficult to state because at the present time there are no serological tests which will aid in the determination of protective antibody concentration other than the unwieldy and expensive monkey protection test.

SUMMARY

Protection tests have been utilized to determine the effect of super-infection upon the potency of immune serum of monkeys with chronic

Plasmodium knowlesi infections. The results of these tests showed that:

1. In 2 groups of monkeys with comparable *P. knowlesi* infections the immune serum of 8 monkeys which had been superinfected on 7 separate occasions over a period of 2 months was much more potent than the immune serum of a group of 7 monkeys which were allowed to continue their chronic course of infection without superinfection.

2. After a series of 9 more intense superinfections the serum from the same 2 groups of monkeys contained no demonstrable protective antibodies.

3. The serum from 8 of the 10 monkeys in the original 2 groups showed a relatively high concentration of protective antibodies following a month's rest and a single superinfection.

4. The results of the experiments indicate that it is possible to increase the potency of immune serum by superinfection, but it is also possible to obtain a decrease in the protective property of the serum by too severe superinfections.

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THE QUANTITATIVE RELATIONSHIP BETWEEN IMMUNE SERUM AND INFECTIVE DOSE OF PARASITES AS DEMONSTRATED BY THE PROTECTION TEST IN MONKEY MALARIA

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In demonstrating the existence of protective antibodies in the serum of *rhesus* monkeys with chronic *Plasmodium knowlesi* infections (1) it was noted that a relatively large amount of immune serum was necessary to produce passive protection and that the use of an excessive number of parasites for the initiation of infection obscured the protective action of the serum. On the basis of these results it seemed important to determine whether a quantitative relationship exists between the amount of immune serum and the dosage of parasites.

The present study is concerned with the determination of the minimal infective dose of *P. knowlesi* in normal *rhesus* monkeys and the effect of varying the number of parasites and the amounts of immune serum used in protection tests. It has been repeatedly demonstrated in many of the infectious diseases that it is impossible to obtain a protective effect from a very potent immune serum if an excess of the infecting agent is used. In malaria, information on this subject is very meagre. Findlay (2) working with malaria in canaries has shown that an increase in the length of incubation time occurred when immune serum and parasites were injected into normal canaries as compared to normal canary serum and parasites. He also noted a progressive increase in incubation time with dilutions of parasites from 1:1 to 1:64.

Materials and Methods

Rhesus monkeys were used exclusively in this work, and all inoculations were made with a strain of *P. knowlesi* which had been under observation in this laboratory since 1933. In order to determine the minimal infective dose, a monkey

with a low grade chronic infection and a parasite count between 10 and 100 per 10,000 red cells was selected. Blood was collected in sodium citrate from the donor animal shortly after sporulation so that all parasites were present in the red cells as young rings. This was done to obviate an increase in the number of parasites in an inoculum due to rupture of segmenting forms. The number of parasites per cubic centimeter of blood was calculated from the red cell count and the parasite count. The citrated blood, immediately after being collected, was diluted in a medium containing equal parts of whole blood from a normal monkey and 2 per cent citrate solution. Results were unsatisfactory when only normal saline solution was used to dilute the infected blood since parasites were destroyed in the higher dilutions. The dilutions used in this study contained 100,000, 10,000, 1,000, 100, 10, and 1 parasites per cc. and 1 parasite per 10 cc. All inoculations were given by injecting 1.0 cc. of each dilution intraperitoneally.

In the experiments designed to show the quantitative relationship between the amount of immune serum and the number of parasites, dilutions of infected blood were made in the same way as in the experiments to determine the minimal infecting dose. The immune serum to be used was obtained from monkeys with long standing chronic infections and pooled so that there was a sufficient amount available for all desired tests. In the first protection experiment the amount of immune serum was kept constant and the number of parasites in the inoculum varied between 1,000,000 and 100. 2 cc. of serum and 1 cc. of each of the dilutions of parasites were incubated together at 37°C. for half an hour before injection, and 2 cc. of serum were administered daily for 9 successive days thereafter.

In the next experiment the same pool of immune serum was used in varying amounts from 2.0 cc. to 0.125 cc., but the number of parasites in the inoculum was kept constant. The monkeys with chronic infections which served as the source of immune serum were bled the second time after a 6 weeks' interval. The pool of serum obtained from this bleeding was set up in one experiment in which there were variations both in the number of parasites and the amount of immune serum.

EXPERIMENTAL

Experiment 1. Determination of the Minimal Infective Dose.—Eight normal monkeys, Nos. 1 to 8 inclusive, were used in this experiment. As shown in Table I, monkey 1 was inoculated with 100,000 *P. knowlesi* parasites, monkey 2 was inoculated with 10,000 parasites, while duplicate monkeys were inoculated with 1,000, 100, and 10 parasites. The results of these inoculations were infection and death in all 8 monkeys. Monkey 1, which received the largest dosage, was the first to show circulating parasites on the 5th day and died on the 9th day. There was a gradual increase in incubation time and the time of death as the number of parasites in the inoculum was decreased. Monkeys 7 and 8, which received the final dilution containing 10 parasites, first showed circulating parasites on the 9th and 13th days and died on the 13th and 17th days respectively.

Since all of the monkeys became infected and died, it was decided to repeat the experiment but to start with 1,000 parasites and dilute

so that the least number was 0.1 parasite (or 1 cc. of a dilution containing 1 parasite per 10 cc.), a theoretical number where infection should not occur.

Nine monkeys were used in the repeat experiment, and, as shown in Table II, monkey 9 was inoculated with 1,000 parasites, monkey 10 was inoculated with

TABLE I

Determination of Minimal Infective Dose of Plasmodium knowlesi Injected Intraperitoneally into Rhesus Monkeys

Monkey No.	Number of parasites injected	Day parasites appeared in blood stream	Results
1	100,000	5th	Died on 9th day
2	10,000	6th	Died on 10th day
3	1,000	7th	Died on 12th day
4	1,000	7th	Died on 11th day
5	100	7th	Died on 12th day
6	100	8th	Died on 13th day
7	10	9th	Died on 13th day
8	10	13th	Died on 17th day

TABLE II

Determination of Minimal Infective Dose of Plasmodium knowlesi Injected Intraperitoneally into Rhesus Monkeys

Monkey No.	Number of parasites injected	Day parasites appeared in blood stream	Results
9	1,000	6th	Died on 12th day
10	100	7th	Died on 14th day
11	10	8th	Died on 11th day
12	10	9th	Died on 14th day
13	1	11th	Died on 18th day
14	1	—	No infection
15	0.1*	—	No infection
16	0.1*	—	No infection
17	0.1*	—	No infection

* 1 cc. of a dilution containing 1 parasite per 10 cc.

100 parasites, monkeys 11 and 12 were each inoculated with 10 parasites, monkeys 13 and 14 were each inoculated with 1 parasite, while monkeys 15, 16, and 17 were each inoculated with 1 cc. of a dilution containing 1 parasite per 10 cc. The results of this experiment showed that the monkeys inoculated with 1,000, 100, and 10 parasites and monkey 13, which received 1 parasite, all became infected and died of acute malaria. Monkey 14, which received an inoculum

diluted so as to contain 1 parasite, and monkeys 15, 16, and 17, injected with 0.1 parasite, failed to develop infections.

The characteristic feature of the above experiments was the progressive increase in the incubation period as the monkeys received a decreasing number of parasites. However, following the detection of microscopically visible parasites, the severity of the disease course and the length of time until death (usually 4 to 5 days) were approximately the same in all animals that became infected.

Experiment 2. Effect of Variation in the Number of Parasites with a Constant Amount of Immune Serum.—Six normal monkeys, Nos. 18 to 23 inclusive, were used in this experiment. Five separate 2 cc. samples of the pooled immune serum were incubated with 1,000,000, 100,000, 10,000, 1,000, and 100 *P. knowlesi* parasites for half an hour at 37°C. Each of these mixtures was then injected intraperitoneally into individual monkeys while a control monkey received 1,000,000 parasites incubated with 2 cc. of normal monkey serum. The immune serum was injected daily in 2 cc. amounts for 9 consecutive days in all except the control animal.

In the results of this protection test, as shown in Table III, 2 monkeys, Nos. 18 and 19 which received the greatest number of parasites, became infected and survived. Monkey 18 had a more severe disease course than monkey 19 which received 10 times fewer parasites. Monkeys 20, 21, and 22, injected with 10,000, 1,000, and 100 parasites respectively, failed to become infected, while control monkey 23 died on the 8th day following inoculation. Since monkeys 20, 21, and 22 failed to become infected, it was considered necessary to show that they were not refractory to infection. These animals were reinoculated 1 month after their original inoculation, each with the same number of parasites that had been used previously. They all became infected and died during the acute attack of malaria.

From this experiment 10,000 was selected as the test dose of parasites that should result in survival with mild infection or no infection when used with 2 cc. amounts of the same pool of immune serum. By decreasing the amount of serum given daily after injecting 10,000 parasites it should be possible to reach an end point where no protection would occur. Accordingly, the following experiment was set up.

Experiment 3. Effect of Variation in Amount of Immune Serum with a Constant Number of Parasites.—The immune serum used in this experiment was taken

from the same pool used in the preceding test. The serum was divided into amounts of 2.0, 1.0, 0.5, 0.25, and 0.125 cc., and each sample was incubated at 37°C. for half an hour with 10,000 parasites. Five monkeys, Nos. 24 to 28 inclusive, were inoculated intraperitoneally with the individual mixtures of immune serum and parasites, while a control monkey, No. 29, was injected with

TABLE III

Protection Experiment with Constant Amount of Immune Serum and Varying Number of Plasmodium knowlesi Parasites in Inoculum

Mon-key No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
18	2 cc. daily for 10 days	1,000,000	13th	Survived, severe infection
19	2 cc. daily for 10 days	100,000	11th	Survived, mild infection
20	2 cc. daily for 10 days	10,000	—	No infection
21	2 cc. daily for 10 days	1,000	—	No infection
22	2 cc. daily for 10 days	100	—	No infection
23	Control, no serum given	1,000,000	4th	Died on 8th day

TABLE IV

Protection Experiment with 10,000 Plasmodium knowlesi Parasites and Varying Amounts of Immune Serum
(Same Serum as Used in Table III)

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
24	2 cc. daily for 10 days	10,000	—	No infection
25	1 cc. daily for 10 days	10,000	12th	Survived
26	0.5 cc. daily for 10 days	10,000	8th	Died on 17th day
27	0.25 cc. daily for 10 days	10,000	8th	Died on 13th day
28	0.125 cc. daily for 10 days	10,000	11th	Severe infection, survived
29	Control, no serum given	10,000	5th	Died on 8th day

10,000 parasites incubated with 2 cc. normal monkey serum. The immune serum was then given daily for 9 days to the 5 monkeys in the same amount as had been used in the incubation with parasites.

As shown in Table IV, monkey 24, which received the most immune serum (a total of 20 cc.), did not become infected.   Monkey 25, which

received 10 cc. of serum, became infected but survived. Monkey 26, which received 5 cc. of serum, died on the 17th day following inoculation. With this animal there was a lengthening of the incubation period and the duration of the acute infection, indicating a slight protective action of the serum. Monkey 27, which received only 2.5 cc. of serum, died on the 13th day. Monkey 28, which received 1.25 cc. of serum during the course of 10 days, had a severe infection but survived. Control monkey 29 died on the 8th day.

From this and the preceding experiment it is evident that the occurrence of infection and the survival or death of the animal in protection tests depend both upon the amount of immune serum and upon the number of parasites in the inoculum. A greater degree of protection was demonstrated either by increasing the amount of immune serum or by decreasing the number of parasites.

Experiment 4. Protection Test Varying Both the Amount of Immune Serum and the Number of Plasmodium knowlesi Parasites.—The immune serum for this experiment was obtained by bleeding the same monkeys which furnished the serum for Experiments 2 and 3. A 6 weeks' interval had elapsed between the two bleedings. There was sufficient quantity of pooled serum to test the effect of variations in the amounts of immune serum and numbers of parasites in the same experiment. As seen in Table V, 5 monkeys in each of 3 groups received immune serum in amounts of 2, 1, 0.5, 0.25, and 0.125 cc. respectively. In the first group 1,000 parasites were incubated separately with the 5 different amounts of immune serum, and each mixture was injected into individual monkeys, Nos. 30 to 34 inclusive. Similarly, monkeys of the second group, Nos. 35 to 39 inclusive, each received 100 parasites, and those of the third group, Nos. 40 to 44 inclusive, 10 parasites. The immune serum was then given daily for 9 days in the amounts indicated, making a total of 20, 10, 5, 2.5, and 1.25 cc. for the respective monkeys. There were 4 control monkeys and each received an injection of parasites which had been incubated with 2 cc. of normal monkey serum for half an hour. Monkey 45 was injected with 1,000 parasites, monkey 46 with 100 parasites, and monkeys 47 and 48 with 10 parasites each.

The results of this experiment showed that among the first group of 5 monkeys, Nos. 30 to 34 inclusive, which were inoculated with 1,000 parasites, only 1 monkey survived. This monkey, No. 30, received the greatest amount of immune serum, a total of 20 cc., and failed to become infected. The remaining 4 monkeys, Nos. 31 to 34, died on the 24th, 31st, 18th, and 16th days respectively although No. 32, according to the parasite count, had apparently recovered but relapsed

and died.   Control monkey 45 died on the 12th day with an over-whelming infection.

There was one survival, monkey 36, in the second group of 5 monkeys which were inoculated with 100 parasites.   Monkey 35 appeared to be recovering from the acute attack of malaria as evidenced by

TABLE V

Protection Experiment with Varying Amounts of Immune Serum and Varying Number of Plasmodium knowlesi Parasites in Inoculum

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
30	2 cc. daily for 10 days	1,000	—	No infection
31	1 cc. daily for 10 days	1,000	18th	Died on 24th day
32	0.5 cc. daily for 10 days	1,000	13th	Died on 31st day
33	0.25 cc. daily for 10 days	1,000	10th	Died on 18th day
34	0.125 cc. daily for 10 days	1,000	9th	Died on 16th day
45	Control, no serum given	1,000	6th	Died on 12th day
35	2 cc. daily for 10 days	100	14th	Died on 25th day
36	1 cc. daily for 10 days	100	10th	Survived
37	0.5 cc. daily for 6 days	100	—	Died on 6th day*
38	0.25 cc. daily for 10 days	100	12th	Died on 19th day
39	0.125 cc. daily for 10 days	100	9th	Died on 15th day
46	Control, no serum given	100	7th	Died on 14th day
40	2 cc. daily for 10 days	10	—	No infection
41	1 cc. daily for 10 days	10	—	No infection
42	0.5 cc. daily for 10 days	10	—	No infection
43	0.25 cc. daily for 10 days	10	—	No infection
44	0.125 cc. daily for 10 days	10	10th	Died on 16th day
47	Control, no serum given	10	8th	Died on 11th day
48	Control, no serum given	10	8th	Died on 13th day

* Intercurrent infection.

decreasing numbers in the parasite count but relapsed and died on the 25th day.   Monkey 36 became infected and recovered.   Monkey 37 died with an intercurrent infection before the appearance of any circulating parasites.   Monkeys 38 and 39 died on the 19th and 15th days after inoculation, while control monkey 46 succumbed on the 14th day.

received 10 cc. of serum, became infected but survived. Monkey 26, which received 5 cc. of serum, died on the 17th day following inoculation. With this animal there was a lengthening of the incubation period and the duration of the acute infection, indicating a slight protective action of the serum. Monkey 27, which received only 2.5 cc. of serum, died on the 13th day. Monkey 28, which received 1.25 cc. of serum during the course of 10 days, had a severe infection but survived. Control monkey 29 died on the 8th day.

From this and the preceding experiment it is evident that the occurrence of infection and the survival or death of the animal in protection tests depend both upon the amount of immune serum and upon the number of parasites in the inoculum. A greater degree of protection was demonstrated either by increasing the amount of immune serum or by decreasing the number of parasites.

Experiment 4. Protection Test Varying Both the Amount of Immune Serum and the Number of Plasmodium knowlesi Parasites.—The immune serum for this experiment was obtained by bleeding the same monkeys which furnished the serum for Experiments 2 and 3. A 6 weeks' interval had elapsed between the two bleedings. There was sufficient quantity of pooled serum to test the effect of variations in the amounts of immune serum and numbers of parasites in the same experiment. As seen in Table V, 5 monkeys in each of 3 groups received immune serum in amounts of 2, 1, 0.5, 0.25, and 0.125 cc. respectively. In the first group 1,000 parasites were incubated separately with the 5 different amounts of immune serum, and each mixture was injected into individual monkeys, Nos. 30 to 34 inclusive. Similarly, monkeys of the second group, Nos. 35 to 39 inclusive, each received 100 parasites, and those of the third group, Nos. 40 to 44 inclusive, 10 parasites. The immune serum was then given daily for 9 days in the amounts indicated, making a total of 20, 10, 5, 2.5, and 1.25 cc. for the respective monkeys. There were 4 control monkeys and each received an injection of parasites which had been incubated with 2 cc. of normal monkey serum for half an hour. Monkey 45 was injected with 1,000 parasites, monkey 46 with 100 parasites, and monkeys 47 and 48 with 10 parasites each.

The results of this experiment showed that among the first group of 5 monkeys, Nos. 30 to 34 inclusive, which were inoculated with 1,000 parasites, only 1 monkey survived. This monkey, No. 30, received the greatest amount of immune serum, a total of 20 cc., and failed to become infected. The remaining 4 monkeys, Nos. 31 to 34, died on the 24th, 31st, 18th, and 16th days respectively although No. 32, according to the parasite count, had apparently recovered but relapsed

and died.   Control monkey 45 died on the 12th day with an over-
whelming infection.

There was one survival, monkey 36, in the second group of 5 mon-
keys which were inoculated with 100 parasites.   Monkey 35 appeared
to be recovering from the acute attack of malaria as evidenced by

TABLE V

*Protection Experiment with Varying Amounts of Immune Serum and Varying
Number of Plasmodium knowlesi Parasites in Inoculum*

Monkey No.	Immune serum injections, amount and time	Number of parasites injected	Day parasites appeared in blood stream	Results
30	2 cc. daily for 10 days	1,000	—	No infection
31	1 cc. daily for 10 days	1,000	18th	Died on 24th day
32	0.5 cc. daily for 10 days	1,000	13th	Died on 31st day
33	0.25 cc. daily for 10 days	1,000	10th	Died on 18th day
34	0.125 cc. daily for 10 days	1,000	9th	Died on 16th day
45	Control, no serum given	1,000	6th	Died on 12th day
35	2 cc. daily for 10 days	100	14th	Died on 25th day
36	1 cc. daily for 10 days	100	10th	Survived
37	0.5 cc. daily for 6 days	100	—	Died on 6th day*
38	0.25 cc. daily for 10 days	100	12th	Died on 19th day
39	0.125 cc. daily for 10 days	100	9th	Died on 15th day
46	Control, no serum given	100	7th	Died on 14th day
40	2 cc. daily for 10 days	10	—	No infection
41	1 cc. daily for 10 days	10	—	No infection
42	0.5 cc. daily for 10 days	10	—	No infection
43	0.25 cc. daily for 10 days	10	—	No infection
44	0.125 cc. daily for 10 days	10	10th	Died on 16th day
47	Control, no serum given	10	8th	Died on 11th day
48	Control, no serum given	10	8th	Died on 13th day

* Intercurrent infection.

decreasing numbers in the parasite count but relapsed and died on the
25th day.   Monkey 36 became infected and recovered.   Monkey 37
died with an intercurrent infection before the appearance of any
circulating parasites.   Monkeys 38 and 39 died on the 19th and 15th
days after inoculation, while control monkey 46 succumbed on the
14th day.

In the last group of 5 monkeys, which were inoculated with 10 parasites, there was only one death. Monkey 44, which received 10 daily injections of 0.125 cc. of immune serum, died on the 16th day. The remaining 4 monkeys, Nos. 40 to 43 inclusive, never became infected, while the 2 controls, Nos. 47 and 48, died on the 11th and 13th days respectively.

The outcome of this experiment indicated that the protective effect of corresponding amounts of serum was more marked when the inoculum contained only 10 parasites than when it contained 1,000 parasites. It was also possible to demonstrate in the same experiment that the use of a greater amount of immune serum against a constant number of parasites resulted in a more marked protective effect.

DISCUSSION

The results of this study indicate that *rhesus* monkeys can be fatally infected with *P. knowlesi* by the injection of less than 10 parasites and probably by 1 or 2. This implies a marked lack of natural resistance in normal *rhesus* monkeys to infection with *P. knowlesi*. Increasing the number of parasites in the inoculum shortens the time required for the organisms to multiply to the extent that they become detectable in blood smears. In untreated monkeys the period from the first positive blood examination to the time of death is approximately the same whether 1 parasite or 100,000 parasites have been injected. A small dose of parasites does not result in a milder disease course by prolonging the incubation time and allowing the host more opportunity to develop active immunity.

It appears from the protection experiments described in this paper that the smaller the number of parasites in the inoculum, the smaller the amount of immune serum required to save the life of the animal. This is known to be true only when the immune serum is incubated with the parasites before injection and is given daily during the incubation period and the first stages of the infection. Once the blood becomes positive for parasites, it is more difficult to protect the animal with immune serum (1). It is probable that if the immune serum were given daily after the first appearance of parasites in the blood, the amount required for protection would be independent of the

number of parasites originally injected.   No experiments of this sort have yet been done.

It is possible that incubation of the parasites with immune serum may have reduced the number of infective parasites in the inoculum. Hence the protective action of the serum may be a result of combined *in vitro* and *in vivo* effects.   In Table V, for example, monkeys 40, 41, 42, and 43 received 10 parasites and developed no infection, while monkey 44 became infected and died at approximately the same time as the controls.   The immune serum in amounts of 0.25 cc. or more may have been sufficient to make the 10 parasites in the inoculum non-infective.   On the other hand, monkey 35, which received 100 parasites and 2 cc. of immune serum, became infected while monkey 30, which received the same amount of immune serum and an inoculum 10 times as large (1,000 parasites), did not become infected. This difference must be ascribed to a greater *in vivo* protective effect of the serum in monkey 30 than in monkey 35 due to individual variations.   Other examples where the *in vitro* and *in vivo* effects of the serum have apparently combined to prevent infection can be seen in monkeys 20, 21, and 22 in Table III.

In Experiments 2 and 3 in which the amount of serum was kept constant and the number of parasites varied, or *vice versa*, consistent results usually were obtained.   An exception was noted in monkey 28 (Table IV) which received 0.125 cc. of immune serum and recovered despite a severe infection, while monkeys 26 and 27 died although they received more immune serum and the same dose of parasites. Another exception is seen in Table V, monkeys 35 and 36.   This indicates that individual variations in monkeys may sometimes adversely affect the expected results.

SUMMARY

The minimal infective dose of *Plasmodium knowlesi* for *rhesus* monkeys was found in this study to be between 1 and 10 parasites when injected intraperitoneally.   As the dose of parasites is increased, the length of time prior to the appearance of circulating parasites is decreased.   However, the severity of the infection once it is established is independent of the initial dose of parasites.

In passive protection experiments a quantitative relationship was

demonstrated between the number of parasites in the inoculum and the effective amount of immune serum given at the time of infection and in equal doses daily for 9 days thereafter. The smaller the inoculum, the less the quantity of immune serum required to prevent the death of the animal.

When relatively large amounts of immune sera and small numbers of parasites were used in the protection experiment, infection was prevented.

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FATE OF NASALLY INSTILLED POLIOMYELITIS VIRUS IN NORMAL AND CONVALESCENT MONKEYS WITH SPECIAL REFERENCE TO THE PROBLEM OF HOST TO HOST TRANSMISSION

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From an epidemiological point of view it is essential to know what happens to poliomyelitis virus which finds its way into the nose of a normal, susceptible animal and of one which is resistant by virtue of a previous attack of the disease. This question has not been investigated experimentally hitherto because until recently nasal instillation of poliomyelitis virus in monkeys caused the disease infrequently and irregularly. It now appears quite probable that of the various neural connections of the nasal mucosa (olfactory, trigeminal, sympathetic, and parasympathetic) poliomyelitis virus instilled into the nose of *Macacus rhesus* monkeys can invade the CNS (central nervous system) only along the olfactory pathway since mechanical interruption of this pathway prevents the development of the disease, as was shown in experiments reported simultaneously by Brodie and Elvidge (1) and Schultz and Gebhardt (2).[1] Additional support is found (*a*) in the early observation of Flexner and Clark (5) that in a monkey sacrificed 48 hours after having its nose swabbed with virus, the olfactory lobes contained the virus at a time when the medulla and spinal cord were not yet infectious, (*b*) in the experiments of Faber and Gebhardt (6) on the progression of nasally instilled virus, and (*c*) in the pathological

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[1] See also Lennette and Hudson (3) and Howe and Ecke (4) for confirmation of these findings. The nature of the operation performed by Howe and Ecke, *i.e.*, section of the olfactory tracts by a lateral approach through the medial orbital walls, was such that it probably avoided the minute and little known nervus terminalis or 13th cranial nerve; the failure of their monkeys to develop paralysis may suggest, therefore, that this nerve also does not ordinarily supply a pathway for this virus.

studies of Sabin and Olitsky (7).   But while it is thus known by what neural route virus in the nose invades the CNS of monkeys, nothing whatever is known about its fate locally in the nasal mucosa, *i.e.*, whether or not, or to what extent it multiplies in it before invading the CNS; how soon such multiplication begins and for how long it continues; how long the virus persists once the infection is established; and other related questions which are of obvious importance when one considers the nasal and associated passages as the sites from which infection may be disseminated in nature.   For the same reason it is essential to know to what extent animals which have once had the disease can carry the same strain of virus as a result either of the first attack or of subsequent nasal instillations.   While the results obtained in monkeys need not represent the course of events in human beings, they may, nevertheless, help in correlating the behavior of the virus in the only available experimental animal with the manifestations of the disease in man.

The literature records but a single attempt to determine the fate of virus applied to the nasal mucosa, that of Flexner and Amoss (8) at a time (1920) when the number of monkeys which developed paralysis with this mode of infection was quite small (8).   These investigators applied the virus by means of a cotton plug which was left in the naris for periods varying from 2 to 24 hours, and sacrificed a number of monkeys at varying intervals after removal of the plug to test for the presence of virus in the excised nasal mucosa.   One monkey was tested after 40 hours, two after 60 hours, and one each at 88 hours, 8 days, and 16 days. Virus was found in the nasal mucosa of only one of these animals, and that was in one of the two sacrificed at 60 hours.   None of the monkeys exhibited any signs of disease at the time they were killed and in two of them (the 88 hour and 16 day animals) in which the olfactory lobes and other parts of the CNS, in addition to the nasal mucosa, were tested for virus, none was found.   These results were interpreted as indicating that the nasal mucosa of certain normal monkeys possesses the power to destroy or otherwise render ineffective the virus applied to it; it was thought that that might, perhaps, be the reason for the failure of many animals to become infected by this route.

In recent years, as a result of further work by Flexner and others, it has become increasingly easy to produce paralytic poliomyelitis in monkeys by nasal instillation of the virus.   The practice has been to give repeated nasal instillations of at least 1 cc. of suspension in each nostril and the incidence of poliomyelitis among monkeys receiving such instillations has gradually risen, for reasons as yet insufficiently clear, from 50 per cent to 75 per cent and in the past 2 years in our hands to 100 per cent (over 100 monkeys), especially when the virus suspensions were

prepared and given in a definite way. When repeated tests showed that one could predict that practically every monkey which received the virus intranasally in a certain way would develop the disease, it was possible to undertake a study of its fate in the nasal mucosa at different stages of the infection. Similarly one could also determine what happened to an infective amount of virus which was given intranasally to monkeys which had recovered from a distinct paralytic attack of the disease.

Methods

Infection of Monkeys by Nasal Route.—It is not yet possible for us to single out the factors to which can be attributed the constancy with which nasal instillation of poliomyelitis virus in monkeys has produced the paralytic disease in our laboratory for the past 2 years. It may, therefore, be worth while to describe in detail the method used and to indicate the effects of certain deviations from it. The virus suspensions are prepared exclusively from the spinal cords and medullae of monkeys which had developed paralysis following nasal instillation of the M.V. strain which has been used only for nasal infection in the past 3 years. Whether or not repeated passage by the nasal route is an essential factor has not been investigated. It appears to be important that the tissue should not have been in glycerol longer than 1 month; in one experiment in which 3 month old cords were used the incidence of successful infection was reduced to 80 to 90 per cent of thirty-six monkeys. Tissue from at least six different monkeys is used to prepare the virus suspension. 10 per cent and 5 per cent suspensions are equally effective but it should be stated that in the preparation of the virus the tissue is first minced finely and ground to a paste with alundum before the necessary amount of diluent is gradually added; after centrifugation at very low speed for only 2 to 3 minutes to remove the alundum and gross pieces, the supernatant liquid and loose sediment are poured off and stirred to yield a milky suspension. The dose is 1 cc. for each nostril instilled with the aid of a pipette fitted with a rubber urethral tip, forcefully expelling the total amount in the direction of the olfactory mucosa (Fig. 1), immediately drawing it back into the pipette, and repeating the process 2 to 3 times. The monkey is then made to take several deep breaths through the nose to aid further in carrying some of the suspension to the olfactory part of the mucosa. It has been the practice for some time to repeat the dose 48 hours later and many of the monkeys used in the present study were infected by this method. We found, however, that the second dose could be given on the same day, either several hours or even a few minutes after the first, and still induce infection in all the monkeys. When, however, the second dose was eliminated in one experiment, so that only 1 cc. per nostril was administered, two of four monkeys failed to develop the disease. Whether the effectiveness of two doses depends upon the larger volume that is administered, increasing the chances of covering more of the olfactory mucosa, or upon the additional virus, has not been determined. The weight and age of the monkeys apparently make little difference, as was pointed out in a previous communication (9).

As is the case with other viruses, so in the intracerebral titration of poliomyelitis virus, there is a minimal dose which is infective for practically all monkeys. When less than this dose is injected, the incidence of infection drops until, with one-tenth of it, there is practically no infection at all. For the M.V. virus, the constantly minimal infective dose by the intracerebral route is in the zone of 0.01 cc. to 0.005 cc. of a 5 per cent suspension.

Fig. 1

One may estimate, therefore, that 4 cc. of a 5 per cent suspension contains about 400 to 1,000 constantly infective minimal cerebral doses. This compares quite favorably with the number of minimal cerebral doses of other viruses which are required to produce infection regularly by the nasal route in the most susceptible animals (9). Thus, with vesicular stomatitis virus in young mice, about 100 to 1,000 minimal cerebral doses are required and with equine encephalomyelitis

virus (also in mice) 1,000 to 10,000 cerebral doses are necessary to produce infection regularly by way of the nose.

Course of Fever in Experimental Poliomyelitis Induced by Nasal Instillation of the Virus.—The clinical course of the disease induced by nasal instillation of the virus differs from that which follows infection by the intracerebral, subcutaneous, and intrasciatic routes in that the period of fever preceding the onset of nervous signs is distinctly longer in the former. When both doses of virus are given on the same day, the first rise in temperature (2°F. or more) occurs in the majority of monkeys 3 or 4 days after nasal instillation. There is, then, a period of at least 3 or 4 days before the appearance of nervous signs, during which the temperature either remains elevated or drops (frequently almost to normal) for a day or two and then rises again, *i.e.*, the dromedary type of curve which is exhibited by about half the number of monkeys.

Preparation of Nasal Mucosa for Inoculation.—The technique employed was influenced by the fact that it was considered important to avoid (*a*) contamination with the olfactory bulbs and (*b*) loss of virus by filtration. The monkeys were exsanguinated and the brain and olfactory bulbs removed. The two fossae harboring the olfactory bulbs were then swabbed out with absorbent gauze and all membranes cut as close to the cribriform plate as possible. The soft tissues were dissected away from the face and the eyes were enucleated to permit cutting through the orbits and maxillary bones for the removal of the nose and palate from the rest of the skull. Further cuts with bone scissors through the nasal bones and palate separated the septum from the other nasal structures and exposed the entire nasal mucosa (olfactory and respiratory). The olfactory portion was cut distal to its connections with the intracranial membranes. The entire mucosa, stripped from the septum, conchae, and lateral walls of the nose, usually weighed about 1 gm. It was finely minced, ground to a paste with alundum, followed by the gradual addition of 10 cc. of physiological salt solution. After horizontal centrifugation at about 2,000 R.P.M. for 10 minutes, the supernatant liquid was spun on the angle centrifuge at 4,000 R.P.M. for 40 minutes. 2 cc. of this supernatant liquid were drawn off for intracerebral injection of a monkey and the rest was thoroughly mixed both with the angle and horizontal centrifuge sediments and used for nasal instillation in the same monkey. Usually there were 7 to 8 cc. of the latter thick suspension which was divided into three portions, one being instilled daily. The nasal mucosa of twenty-two monkeys was prepared in this manner and in only four instances was the intracerebral injection followed by a fatal, bacterial (pneumococcus) meningitis, three cases occurring in a single experiment.

Preparation of Nervous Tissue for Inoculation.—The olfactory bulbs (from either normal or convalescent monkeys) were ground in a mortar without any abrasive and taken up in 1 cc. of saline solution, all of which was injected intracerebrally in a monkey. When other regions of the CNS of convalescent monkeys were tested, several grams of the tissue were ground with alundum and made up to

10 per cent suspension with phosphate buffer of pH 7.4. After very light centrifugation, 12 cc. of the milky suspension was drawn off for intracerebral (2 cc.) and intraperitoneal (10 cc.) inoculation of a monkey. To the remainder of the suspension sufficient phosphate buffer (usually not more than an equal volume) was added to bring the volume up to 100 cc., which was distributed among three U tubes and submitted to cataphoresis.

Cataphoresis.—The technique was essentially that employed by Olitsky, Rhoads, and Long (10), except that 3 per cent agar was used in the bridges and a milliamperage of 2.5 to 5 for a period of 4 to 5 hours.

Controls.—Whenever any of the normal or immune convalescent monkeys ·(which were to be sacrificed in an experiment) received nasal instillations of virus, there were at least three or more other monkeys which were given the same amount of the same suspension of virus and allowed to develop the paralytic disease, as an index of the infectivity of the virus used in each experiment. Daily rectal temperatures were taken on all animals.

<center>EXPERIMENTAL</center>

Sensitivity of Method.—When several tests with nasal mucosa from paralyzed monkeys failed to reveal virus, it became essential to determine approximately how many minimal cerebral infective· doses (M.C.I.D.) had to be present in the nasal mucosa in order to be demonstrable by the method employed.

To the ground up nasal mucosa of a normal monkey was added 0.05 cc. of a Berkefeld N filtrate of a 5 per cent virus suspension (about 5 to 10 M.C.I.D.), and then 10 cc. of saline, the final mixture being submitted to the same horizontal and angle centrifugation as was described under Methods. Another mixture containing 0.2 cc. of the same virus filtrate (about 20 to 40 M.C.I.D.) and the nasal mucosa of another monkey was similarly prepared. Mixtures of 0.05 cc. and 0.2 cc. of virus filtrate each with 10 cc. of salt solution, similarly centrifuged, served as controls. Each preparation was inoculated into a monkey intracerebrally (2 cc.) and intranasally (7 to 8 cc.).

The results shown in Table I indicate that the monkeys inoculated with the mixtures containing nasal mucosa succumbed much more rapidly than those which received equivalent amounts of saline-virus mixtures. It is evident, therefore, that little or no virus is lost during the various steps of the procedure and that when as little as 5 to 10 M.C.I.D. are present in the nasal mucosa, it should be possible to detect the virus.

Virus in Nasal Mucosa and Olfactory Bulbs at Various Intervals after Nasal Instillation in Normal Monkeys.—The object of these tests was

TABLE I

Sensitivity of Method for Detecting Poliomyelitis Virus in Normal Nasal Mucosa

Medium	Amount of virus added	Dose and route of inoculation	Monkey No.	Result*
Entire nasal mucosa of normal monkey in 10 cc. saline	0.05 cc. (about 5 M.C.I.D.)	2 cc. intracerebrally	1	Paralysis 5, dead 6
	0.2 cc. (about 20 M.C.I.D.)	8 cc. intranasally	2	" 5, prostrate 6
		" "		
10 cc. saline	0.05 cc. (about 5 M.C.I.D.)	" "	3	Paralysis 15, " 17
	0.2 cc. (about 20 M.C.I.D.)	" "	4	" 11, " 12

* Numbers represent the day following inoculation or first instillation of virus on which paralysis or death occurred.

to determine (a) how much of the nasally instilled virus remained in the nasal mucosa within the first few hours, i.e., the amount of virus which may be present without local increase; (b) whether or not the virus multiplied or increased in the nasal mucosa and in case it did, whether or not it occurred before invasion of the CNS; (c) how soon virus could be detected in the olfactory bulbs, particularly in relation to the time when fever first appeared and paralysis developed; (d) for how long a period virus could be detected in the nasal mucosa after the CNS had become invaded.

Thirteen monkeys were sacrificed at different stages of the experimental disease, beginning with 4 hours after nasal instillation and including animals which were completely paralyzed on the 8th or 9th day. The nasal mucosa and olfactory bulbs were tested for virus as described under Methods, and the results are shown in Table II.

It appears from these data that within 4 hours after nasal instillation of at least several hundred M.C.I.D. of virus, less than 5 to 10 M.C.I.D. remain in the entire nasal mucosa since no virus was detected by subinoculation. This finding is in accord with our observations on nasally instilled vesicular stomatitis virus in mice (11) and guinea pigs (12), in which no virus could be detected in the nasal mucosa even 1 to 3 hours after nasal instillation of as much as 100,000 M.C.I.D. Since one can be reasonably certain that animals in which the virus disappears in this manner would have succumbed, the question arises whether the subsequent infection develops from an undetectably small amount which becomes fixed to the cells of the nasal mucosa (although a relatively large amount is necessary to initiate a successful take), or whether there is, perhaps, an early non-infective stage in the attack of virus upon cells—a question which at the present state of our knowledge cannot be answered one way or another.

At 24 and 48 hours virus was still undetectable in the nasal mucosa and olfactory bulbs, the latter apparently still containing less than a single M.C.I.D., since both bulbs were transferred practically without loss into the brain of another monkey. In both monkeys which were sacrificed 3 days after nasal instillation virus was found both in the nasal mucosa and the olfactory bulbs. The animals inoculated with the nasal mucosa did not develop paralysis until the 15th or 13th day,

TABLE II

Tests for Virus in Nasal Mucosa and Olfactory Bulbs at Various Intervals after Nasal Instillation in Normal Monkeys

Time after nasal instillation of poliomyelitis virus	Monkey No.	Fever or paralysis	Effect of inoculating monkeys with	
			Nasal mucosa	Olfactory bulbs
4 hrs.	1	None	0	n.t.*
24 "	2	"	Bacterial contamination	0
24 "	3	"	0	0
2 days	4	"	Bacterial contamination	0
2 days	5	"	0	0
3 "	6	1st day of fever (105.6°F.)	Paralysis 15th day (poliomyelitis histology)	Paralysis 5th day
3 days (after 1st dose) (1 day " 2nd ")	7	None	Paralysis 13th day (poliomyelitis histology)	" 6th "
4 days (after 1st dose) (2 " 2nd ")	8	Fever 3rd and 4th days	0	Paralysis 5th day
7 days (after 1st dose) (5 " 2nd ")	9	Fever since 4th day	Bacterial contamination	" " " .
8 days	10	Paralyzed 7th day	0	" " "
8 days	11	" "	0	" " "
8 days	12	" "	0	n.t. "
9 days	13	" "	0	" "

* n.t. = not tested.

as compared to 5 or 6 days in the monkeys inoculated with the olfactory bulbs from the same animals. The clinical course of the disease in the monkeys inoculated with the nasal mucosa as well as the histological examination of their olfactory bulbs suggested that they succumbed as a result of the intracerebral inoculum (*i.e.*, 1/5 of the total nasal mucosa) and not as a result of the remainder of the material instilled intranasally. Sections of small pieces of the olfactory bulbs of one of the monkeys sacrificed on the 3rd day revealed distinct pathological changes consisting of acidophilic necrosis of a number of mitral cells and an infiltration of the outer layers of the bulbs with mononuclear and polymorphonuclear cells (7). It is also noteworthy that the first detection of virus in the nasal mucosa and olfactory bulbs corresponded to the first appearance of fever in one monkey but was unassociated with any rise in temperature in the other. In monkeys sacrificed later than the 3rd day after nasal instillation, virus was demonstrable in the olfactory bulbs but not in the nasal mucosa. It may be interesting to recall here that in the experiments of Flexner and Amoss (8), already described in a preceding section, the one positive result in recovering virus from the nasal mucous membranes was 60 hours after the administration of the virus by means of a cotton plug.

It appears, therefore, that within a few hours of and for 2 days after the nasal instillation of an amount of poliomyelitis virus which is capable of inducing paralysis in practically all monkeys, none can be detected in the excised nasal mucosa. It then becomes demonstrable simultaneously in the olfactory bulbs and nasal mucosa on the 3rd day, and while it subsequently remains in the bulbs and progresses through the rest of the CNS to produce the complete paralytic disease, it again either disappears from the nasal mucosa or diminishes in amount to such an extent that it cannot be again recovered. One cannot be certain whether the transitory presence of detectable, though small, amounts of virus in the nasal mucosa on the 3rd day is the result of local multiplication in the olfactory neurons of the first order or of an overflow of virus multiplying in the olfactory bulbs, although the failure to find it in the mucosa on the 4th day or later at a time when the bulbs were highly infective may, perhaps, be considered as evidence against the latter assumption. The present data are also sig-

nificant in showing that virus and lesions may be demonstrable in the CNS (at least the olfactory bulbs) before the onset of fever, and that the interval between involvement of the bulbs and development of paralytic signs is about 4 to 5 days. It is furthermore evident that the nasal mucosa of susceptible monkeys is not a site where the virus of poliomyelitis can lodge passively in any appreciable amount and that even when it becomes demonstrable for a single day during the entire experimental disease, the amount present is so small that if the entire nasal mucosa were instilled intranasally into another monkey it would be insufficient to infect it.

Although the numerous unsuccessful attempts to transmit poliomyelitis to monkeys by contact infection were made with animals inoculated by routes other than the intranasal, one can readily understand from the results obtained why contact infection should not occur even with monkeys infected by the nasal route. Such an experiment was, nevertheless, carried out.

Three monkeys were given the usual nasal instillations of poliomyelitis virus and immediately put into a small cage (84 x 71 x 76 cm.) in intimate contact with six normal monkeys. The inoculated monkeys developed the disease in the usual time, while the six contacts exhibited neither fever nor other signs of illness during a 5 week observation period. It may be added that between the 10th and 20th days after the beginning of this experiment, fifteen additional monkeys, intracerebrally injected and in the preparalytic or paralytic stages of the disease, were crowded into the same cage with the normal contacts, without influencing the outcome.

Fate of Nasally Instilled Poliomyelitis Virus in Immune, Convalescent Monkeys.—The main purpose of the following tests was to determine whether animals which are resistant to reinfection by virtue of a previous attack of the disease can act as carriers of the same strain of virus. Secondarily it was of interest to determine whether in such animals nasally instilled virus can invade the CNS as it does in normal monkeys and in the greater number of vaccinated animals whose immunity appears to be limited only to the presence of neutralizing antibodies in their blood (13, 14).

Six convalescent *M. rhesus* monkeys were used in these tests. Four of these had their primary infection following nasal instillation and two following intracerebral injection of the virus (M.V. strain). All had had distinct paralysis as a result of the primary infection, all had received additional virus instillations

TABLE III

Protocols of Convalescent, Immune Monkeys

Monkey No. and History	Date	Treatment	Remarks
43 Dec. 3, 1935, and Dec. 5, 1935, virus intranasally. Paralysis limited to face. Complete recovery	1936 Feb. 1 " 3 → Mar. 5 " 7 → Apr. 13 " 15 → May 11	2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally	4 control monkeys developed poliomyelitis " " " " 12 " " " " " " " " 3 " " " " " " " "
7-31 Jan. 31, 1936, virus intracerebrally. Paralysis of all extremities. Recovery with residual paralysis	Mar. 5 " 7 → Apr. 13 " 15	2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally 2 cc. 10% virus intranasally	3 control monkeys developed poliomyelitis. Sacrificed (4 hrs. later). Nasal mucosa, no virus found Sacrificed (48 hrs. after virus) Nasal mucosa Olfactory bulbs Pons, medulla, and cord, before and after cataphoresis } no virus detected 0.9 cc. 10% CNS suspension failed to neutralize 10 and 20 M.C.I.D. of virus

7-20	Mar. 5	2 cc. 10% virus intranasally	
	" 7	" " " "	
	→	Remained well	
Jan. 2, 1936, and Jan. 4, 1936, virus intranasally. Paralysis of left upper extremity only. Recovered with residual paralysis of left arm	Apr. 13	2 cc. 10% virus intranasally	
	" 15	" " " "	
	→	Remained well	
	May 11	2 cc. 10% virus intranasally	
	" 13	" " " "	
	" 14	Temperature normal; monkey well	Sacrificed
			Nasal mucosa
			Olfactory bulbs } no virus
			Pons, medulla, and cord, before } detected
			and after cataphoresis
			0.8 cc. of spinal fluid failed to neutralize 20 M.C.I.D. of virus

TABLE III—*Concluded*

Monkey No. and History	Date	Treatment	Remarks
7-19	*1936* Mar. 5 " 7 ↓ Apr. 13 " 15 " 17	2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally " " " " Temperature normal; monkey well	Sacrificed Nasal mucosa Olfactory bulbs Hypothalamus } no virus detected Pons, medulla, and cord, before and after cataphoresis 0.9 cc. 10 per cent CNS suspension failed to neutralize 10 and 20 M.C.I.D. of virus
Jan. 2, 1936, and Jan. 4, 1936, virus intranasally. Complete paralysis of lower extremities and partial paralysis of upper extremities. Recovered with residual paralysis			
42	Feb. 1 " 3 ↓ Mar. 5 " 7 " 10	2 cc. 10% virus intranasally " " " " Remained well 2 cc. 10% virus intranasally " " " " Temperature normal; monkey well	Sacrificed Nasal mucosa Olfactory bulbs Hypothalamus } no virus detected Pons, medulla, and cord, before and after cataphoresis
Dec. 3, 1935, and Dec. 5, 1935, virus intranasally. Paralysis of right arm and weakness of other extremities. Recovered with residual paralysis of right arm			

34-15		Virus intracerebrally	Intracerebral control—poliomyelitis
	Jan. 3	Remained well	
Dec. 31, 1935, virus intracerebrally and intraperitoneally. Widespread partial paralysis. Recovered with slight residual paralysis	Mar. 5 " 7	2 cc. 10% virus intranasally " " " " " Remained well	
	Apr. 13 " 15 " 20	2 cc. 10% virus intranasally " " " " " Temperature normal; monkey well	Sacrificed Nasal mucosa Olfactory bulbs Hypothalamus Pons, medulla, and cord, before and after cataphoresis } no virus detected 0.9 cc. 10 per cent CNS suspension failed to neutralize either 10 or 20 m.c.i.d. of virus

The control normal monkeys which were given the same amount of virus simultaneously with each test on the convalescents all developed poliomyelitis; the numbers of animals used in the various tests are recorded only in the protocol of M 43.

which they resisted, and all had neutralizing antibodies in their serum at the time of the last test when they were sacrificed. Normal monkeys which were given virus each time any of the convalescent animals received it, invariably succumbed with typical poliomyelitis. All but two of the convalescents had two doses of virus, 48 hours apart, and individual animals were sacrificed at the following intervals for tests on the nasal mucosa and the nervous system: 4 hours after a single dose (M 43), 48 hours after a single dose (M 7-31), 72 hours after the first and 24 hours after the second dose (M 7-20), 96 hours after the first and 48 hours after the second dose (M 7-19), 120 hours after the first and 72 hours after the second dose (M 42 A), and 7 days after the first and 5 days after the second dose (M 34-15). The results are summarized in the protocols presented in Table III.

It is clearly apparent that in none of the six immune monkeys, sacrificed at intervals of 4 hours to 7 days after nasal instillation of amounts of virus which regularly produced the disease in normal animals, was the infective agent demonstrable either in the nasal mucosa or in different parts of the CNS. It should be further noted that neither by direct test nor with the aid of cataphoresis was it possible to demonstrate the presence of virus in the CNS,[2] which points not only to its rapid inactivation relatively early after the appearance of paralysis but also to the inability of newly instilled virus (of the same strain) to invade and multiply in the CNS of such animals. It may be of interest to point out in this respect that (a) tests with extracts of the nasal mucosa of two of these monkeys failed to neutralize completely 10 M.C.I.D. of virus (Table IV), (b) tests with suspensions of CNS of three of the convalescent monkeys failed to reveal that the nervous tissue of these resistant animals had any capacity to inactivate or inhibit the effects of small amounts of virus, and (c) that vaccinated monkeys which possess as much neutralizing antibody in their blood as these convalescent animals (16) are not, as a rule, resistant to similar amounts of nasally instilled virus.

Apart from these considerations, it is clear that monkeys which are resistant by virtue of a previous attack of the disease quickly rid

[2] The demonstration by means of cataphoresis of virus in a single monkey 23 days after infection has been brought forth by some investigators as evidence that persistence of immunity in poliomyelitis may be correlated with persistence of virus. The present tests, taken together with those performed by Levaditi and Lepine (15), indicate that as regards experimental poliomyelitis there is no evidence for such an assumption.

themselves of virus which may again be introduced on their nasal mucosa, and thus cannot act as carriers (or be a source of infection) of the same strain of virus which caused the primary infection. The fact that this applies only for the same strain of virus is stressed because there seems to be little doubt now that monkeys which have recovered from a distinct paralytic attack with one strain of poliomyelitis virus, and are resistant to further inoculations with the same strain, can be reinfected by the nasal instillation of another strain of

TABLE IV

Effect of Extracts of Nasal Mucosa from Normal and Immune, Convalescent Monkeys on Small Amounts of Poliomyelitis Virus

Nasal mucosa of	Amount of extract	Amount of virus (about 10 M.C.I.D.)	Monkey injected intracerebrally with incubated mixture	Result*
	cc.	*cc.*		
Normal monkey A	0.9	0.1	1	Paralysis 5, dead 6
" " B	"	"	2	Bacterial meningitis
Convalescent, immune monkey C	"	"	3	Paralysis 16, prostrate 16
" " " D	"	"	4	" 16, " 17
Control Serum of normal monkey A	0.9	0.1	5	Paralysis 7, prostrate 8

* Numbers as in Table I.

virus. Monkeys convalescent from and resistant to the M.V. strain have been shown to develop typical poliomyelitis a second time following the nasal instillation of the Philadelphia (1932) strain of the virus (17).[3]

While probably it would be generally agreed that an occasional convalescent monkey may not be resistant to reinoculation with the homologous strain of

[3] Personal communication by Dr. Flexner; this also occurred in five of six such animals which we transferred to Dr. Flexner for further study.

virus (18), a review of the literature indicates no concurrence on the rarity or frequency of such lack of resistance. In 1910, Flexner and Lewis (19) showed that none of ten previously paralyzed monkeys developed a second attack upon intracerebral reinoculation. In the protocols of a paper by Schultz, Gebhardt, and Bullock, 1931 (20), it appears that of twelve monkeys having had partial or complete paralysis of one or more extremities following intracerebral virus inoculation, all resisted further repeated intracerebral injections of homologous virus. Paul and Trask (21) stated that they failed to reinfect thirteen convalescent monkeys by intracerebral injection of the homologous strain of virus and concluded that "as others have often shown, such instances of reinfection must be uncommon." In 1936, Jungeblut (22) pointed out that of thirty-four animals with a previous history of distinct paralysis, all were refractory to intracerebral reinjection of 1 cc. of 10 per cent homologous virus suspensions (Aycock strain); of twenty others with a history of a febrile cycle following intracerebral injection of virus and certain inactivating agents, all failed to resist similar reinoculation. In the same year, Toomey (23) indicated that animals which received injections of virus into the intestine or brain and developed only paresis or limited palsies, contracted distinct paralytic poliomyelitis after reinjection with homologous virus (1 to 2 cc., 10 per cent M.V. suspensions), while animals that had severe quadriplegia seemed to be protected. In 1936 we described (16) nine monkeys convalescent from paralytic poliomyelitis (one or more extremities) which were given nasal instillation of homologous, M.V. virus, capable of inducing the disease in practically all normal animals. Six of the nine resisted repeated instillation (these are included in the present paper), while three became prostrate and died within 3 to 4 days; two of these monkeys were investigated and no virus could be demonstrated in their CNS and there were no acute pathological changes to indicate a second attack of the disease. Such demonstration of virus and pathological change should, whenever possible, be used to authenticate experimental second attacks.

In 1936 and 1937, Flexner (17), in elaborating his material from 1912 to 1933, described four monkeys with apparent second attacks following reinoculation with homologous virus. Two of the four had had paralysis following intracerebral injection (M.A. strain, 1912–1913) and during the course of repeated subcutaneous injections of virus months later, there was sudden development of paralytic symptoms and death. The third monkey had progressive symptoms of tremor, ataxia, and weak legs, accompanied by fever which followed intracerebral inoculation of M.V. virus; it later succumbed with characteristic poliomyelitis as a result of nasal instillation of the same virus. The fourth monkey had no clinical symptoms but spinal fluid pleocytosis of 335 cells following the first series of five nasal instillations of 1933 virus; it responded to another series of nasal instillations with the same virus given 40 days later, with paralytic poliomyelitis and death. Recently we observed reactions similar to those found in this fourth monkey: Two animals (one previously given immunizing injections of virus) reacted to later instillation of M.V. virus with fever (to 105.2 and to 105°F.), spinal fluid pleocytosis (from 35 to 95 and from 25 to 280 cells respectively), and with ques-

tionable signs of excitement and tremor, but without definite paralysis in either, and succumbed with prostrating paralytic poliomyelitis to a second dose of M.V. virus instilled 25 days later. Recently Kessel and Stimpert (24) stated that of four monkeys recovered from infection with M.V. virus, two developed a second attack after reinoculation with the homologous strain.

DISCUSSION

When poliomyelitis virus is instilled into the nares of normal monkeys, the greater portion of it can be seen to flow down into the throat and mouth and to be swallowed in short order. It is evident, however, from what has been said before, that enough must remain or be taken up by the nasal mucosa, and probably more specifically by the olfactory portion of it, to initiate the course of events which now leads almost constantly to the invasion of the CNS by the olfactory pathway. Yet, within 4 hours and for at least 48 hours after nasal instillation, no virus was demonstrable either in the entire excised nasal mucosa or in the olfactory bulbs. The sensitivity of the method was such that if about five minimal cerebral infective doses (M.C.I.D.) were present in the mucosa and one in the bulbs, it should have been possible to detect the virus. The first appearance of virus in demonstrable amounts in the nasal mucosa was about 72 hours after the first instillation and almost simultaneously with the first demonstration of virus and lesions in the olfactory bulbs. Subsequently, however, and as early as the 4th day after nasal instillation, while the virus remained in considerable quantities in the olfactory bulbs and was spreading elsewhere in the CNS, the nasal mucosa was no longer infective by the method employed. Repetition of this work on a larger scale, or with different quantities of virus, may, perhaps, modify the particular time relationship noted here, but the principle that the nasal mucosa is infective for a short and transitory period during the experimental disease induced by nasal instillation of virus appears quite clear. It is, furthermore, noteworthy that in the monkey the infectivity of the nasal mucosa during that transitory period is of such a low order that it is demonstrable by intracerebral inoculation only after a prolonged incubation period and probably could not infect another monkey by the nasal route even if the entire nasal mucosa were transferred to it. That virus is not demonstrable in the blood or spinal fluid of monkeys at various times after nasal instillation has

already been shown by Brodie and Elvidge (25) and as regards blood, by the extensive transfusion experiments of Gordon and Lennette (26). In view of all these observations, it was not surprising to find that experimental poliomyelitis induced by nasal instillation of the virus does not spread spontaneously to other monkeys.

The hypothesis that in the human disease the virus first attacks the olfactory mucosa and that it is from this site also that it is disseminated from man to man would require that a great deal more virus be produced locally than occurs in the monkey, or that each monkey infective unit be equivalent to more than 100 or 1,000 infective units of virus in man. The relatively low incidence of positive results obtained in attempts to demonstrate virus in the nasal washings of human cases, even during the acute stage of the disease, could be due, in addition to the presumable difficulties of experimental transmission, to the possibility that in the majority of human cases as in monkeys, the nasal mucosa was most infective before the disease became clinically apparent.

It is of interest to note in this connection that Taylor and Amoss (27) obtained virus from the nasal washings of a child 5 days before the onset of clinical signs of poliomyelitis and that in a large series of abortive cases studied by Paul and Trask (28), and Paul, Trask, and Webster (29), the three positive isolations of virus were all from cases on the 1st day of the clinical disease. One can find satisfactory reports of perhaps nine additional isolations of virus in the period of the 4th to the 17th days of the disease (see recent summary by Stillerman and Brodie, 30), but when one considers the total number of nasal washings that were examined to obtain these results, it is not improbable that so late a persistence of virus may perhaps be exceptional. The nasal mucosa itself has been studied by Flexner and Amoss (31) in three human beings dying during the 1st week of the disease, and virus was isolated from one of the three. In the same investigation they recovered the virus from the tonsils of five of ten patients dying within the 1st week of the disease, in one instance the tonsils yielded virus while the nasal mucosa from the same case did not, and in another virus was obtained from the nasal mucosa but not from the tonsils. Flexner and Clark (32), at an earlier date, reported that with unfiltered, phenolated suspensions they obtained virus from the tonsils of each of four human cases. It is important to recall here that the tonsils form part of a chain of lymph nodes into which the lymphatics from the nasal mucosa drain. It may be of interest in this respect that in guinea pigs the cervical lymph nodes draining the nasal mucosa were found to contain no vesicular stomatitis virus several hours after nasal instillation of 50,000 to 500,000 M.C.I.D. and little or none at the height of local (nasal) multiplication about 2 days later (12) but in the supervening few days, when the virus had practically disappeared

from the nasal mucosa, it was easily demonstrable in these lymph nodes (unpublished observations).

The studies on the convalescent, immune monkeys clearly showed that after nasal instillation of the same strain of virus, it was quickly "washed" away as in normal animals, but it failed to reappear later or to invade the CNS as is the case in normal monkeys. That the humoral antibodies probably do not determine this result is evident from the observations that vaccinated monkeys possessing the same amount of neutralizing antibody as convalescents (16) usually succumb when given the same amount of virus, as well as from the fact that monkeys, immune as a result of a previous paralytic attack of the disease, are resistant to subsequent nasal instillation of virus long before the antibodies become demonstrable in the serum (16). As regards the possible effect that neutralizing antibodies might have on the capacity to detect the virus in the nasal mucosa or CNS, it was shown that these tissues did not contain enough to mask the presence of as little as 10 m.c.i.d. It is important to stress here that this applies only to reinoculation with the same strain and to monkeys which have suffered a paralytic attack of the disease. The possible significance of these data from an epidemiological point of view is in the suggestion that individuals who are immune because of a previous attack of the disease (not to be confused with natural resistance which perhaps determines whether an attack of poliomyelitis will be apparent or inapparent) may no longer act as transmitters of the same strain or type of virus infection.

SUMMARY

With a method of intranasal instillation of poliomyelitis virus that brings about infection of all *M. rhesus* monkeys subjected to it, a study was undertaken of the fate of nasally instilled virus in normal and convalescent, immune animals. Control experiments revealed that nasal mucosa of normal monkeys contained no observable antiviral factors and that when five or ten minimal cerebral infective doses were added to the mucosa, virus could be detected by the employed procedure. In the olfactory bulbs even a single infective dose could be recovered, since suspensions of both bulbs could be transferred to the brain of a monkey without any loss of material.

After nasal instillation of virus in normal monkeys, it disappeared

quickly (4 hours or less) and could be recovered neither from the excised nasal mucosa nor from the olfactory bulbs during the first 48 hours. At 72 hours, just before or coincident with the first rise of temperature, virus was found in very small amounts in the nasal mucosa and for the first time also in the olfactory bulbs. At 96 hours, at least 3 days before the appearance of nervous signs, and later, while virus continued to be present in considerable amounts in the olfactory bulbs (and presumably elsewhere in the central nervous system), none was detected in the nasal mucosa. In convalescent, immune animals receiving the same strain of virus intranasally which caused the original infection, none could be recovered from the nasal mucosa or central nervous system at 4 hours, 1, 2, 3, 4, 5, and 7 days. The bearing of these observations on the problem of host to host transmission of poliomyelitis virus is discussed.

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VITAL STAINING OF THE CONNECTIVE TISSUES

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PLATE 1

When trypan blue is injected intravenously, the animal appears blue within a few minutes, not only in its integument, but in the internal organs as well. 12 or more hours after the administration of the dye, there are innumerable granules within macrophages and other colloidopexic cells, easily demonstrable by ordinary histological technique. But the animal is blue long before such granules are formed. In the extensive literature on vital staining with the acid colloidal dyes, most of the attention has been focused on the formation of intracellular granules and on the problems of the reticulo-endothelial system. The present investigation is concerned with the problem, where, apart from the blood stream, is trypan blue before it is flocculated?

After trypan blue is introduced into the circulating blood, some is excreted almost at once, while much of it passes into the tissues with equal rapidity. Once the dye has passed the endothelium, its further course has not been adequately studied. Petroff (1) found that, in the mesentery of the living frog, the walls of the vessels may become colored in less than 20 minutes after the dye is administered. He concluded that such staining of the vessel walls is due to the specific capacity of the elastic elements to adsorb the dye in question. In this and several similar investigations (2–6) interest has focused chiefly on the passage of substances directly from the blood through the endothelium of the larger blood vessels, and on the possible relations of these data to the problems of atherosclerosis. Apart from the vital staining of the elastic tissue in the blood vessel walls, however, the locus of the dye, before the formation of granules, is not satisfactorily explained. Cappell (7), for example, says that, after an intravenous injection, when the animal becomes blue in a few minutes, "thorough microscopic examination of the tissues at this time shows no evidence of the dye, which is located in the body fluids."

Methods

In the present study, guinea pigs and white mice were used. Trypan blue was administered intravenously, in varying dosages, and tissue was examined from 10

63

minutes to 72 hours after the injection. In some instances small portions of tissue were placed directly into glycerin, and examined immediately under the microscope. This sufficed to show all the principal features, but for more detailed study fixation and sectioning were necessary. Several fixatives were tried, but by far the most satisfactory was the Heidenhain's Susa mixture (formalin 20, corrosive sublimate 5, NaCl 0.5, acetic acid 4, trichloracetic 2, aq. 80). Fixation for 2 hours was adequate. The tissue, without washing, was then rapidly dehydrated in several changes of 95 per cent and absolute alcohol within a period of 4 to 5 hours, cleared in chloroform, and embedded in paraffin. Sections were cut at 30 microns, and, after removal of mercury precipitate, were examined without counterstain. Nuclei could be very readily seen by closing down the substage diaphragm.

In the study of the brain, the method of Spalteholz was utilized in some instances. The brains (entire) were fixed for 3 hours at 37°C. in absolute alcohol, followed by 2 to 3 hours in absolute-benzol, cleared in benzol overnight, and in oil of wintergreen the next day.

Mice proved to be far more satisfactory than guinea pigs for intravenous injection of trypan blue. 5 mg. of the dye (0.5 cc. of a 1 per cent solution) are supported by an 18 gm. mouse without difficulty, and produce a deep coloration. A 300 gm. guinea pig, on the other hand, has often succumbed within an hour to 15 mg. of the dye, even when injected very slowly. Even if it survived, the resulting coloration was not intense. To produce a satisfactory degree of vital staining in a guinea pig with a single injection, 30 to 40 mg. are necessary, and in the great majority of cases this results in death within a few hours or less. At such time the features described below are vividly discernible, but in order to compare such findings with those in animals 24 to 72 hours after injection, mice were used for the most part.

In mice, on different occasions, as much as 15 mg. have been given intravenously (0.75 cc. of a 2 per cent solution) without causing death. Very rarely a mouse receiving 5 mg. intravenously dies 24 to 36 hours after injection, but this is in marked contrast to the behavior of guinea pigs, in which death occurs in a few hours.

Many of the animals had previously been used in experiments with equine encephalomyelitis, but had survived without symptoms. The findings in such survivors, however, were identical in every respect with those in normal animals.

Out of the large number of mice and guinea pigs studied, a series of 30 mice, 5 to 6 weeks of age, and about 17 to 19 gm. in weight, were treated in the following manner. Half were given 2.5 mg. of dye intravenously, the other half 5 mg. Animals were sacrificed at intervals of 10 minutes, 1, 4, 24, and 72 hours. The following organs were prepared for examination as described above: bladder, small intestine, mesentery, kidney, liver, spleen, diaphragm, chest wall, lung, aorta, esophagus and trachea, ear, and brain. Practically all the other organs have been studied at one or another time, but for systematic observation the foregoing seemed an adequate selection. The coloration of animals receiving the smaller dose

differed only in intensity. 2.5 mg. of dye were adequate to show all the features described below, but twice that dose made examination and photography simpler.

General Features of the Staining

A mouse of 18 gm., examined 10 minutes after the intravenous injection of trypan blue, already shows the fixation of the dye by the intercellular connective tissue elements. These elements are three in number,—collagen, reticulin, and elastic tissue.

Collagen.—Union of dye and tissue is most brilliantly in evidence in hollow viscera with considerable connective tissue beneath the epithelium, that is, with a well developed lamina propria; *e.g.*, the urinary or gall bladder, ureter, vas deferens, esophagus, to a lesser extent the intestines, and the like. Such organs are, macroscopically, a deep blue. Microscopic examination (Fig. 1, esophagus) shows an unstained epithelium but a very deeply stained tunica or lamina propria. The muscle fibers themselves are colorless, although connective tissue septa stand out with a prominence proportional to their size. The adventitia stains, but somewhat less intensely.

A second example, here illustrated (Fig. 3), is the abdominal wall of a mouse. (The anterior abdominal wall was rolled up before fixation, and the architecture is consequently distorted.) The fascial planes are intensely blue, while the connective tissue septa are stained somewhat less deeply. Excellent preparations may also be made from the diaphragm, where the pleural and peritoneal surfaces stand out vividly against the colorless muscle fibers.

The connective tissue partitions of all the organs are stained in a similar fashion. Such glands as the thyroid, submaxillary, or testis, with abundant collagen, or the liver, with proportionally less, as well as the connective tissue coats of the hollow viscera or of the skin, all exhibit the same selective vital staining of the collagen. This staining is diffuse and not particulate or granular. The collagen bundles are stained in their entirety.

This staining of collagen is independent of the density of the tissue or the degree of vascularization. The compact connective tissue, such as dura mater, capsule of the kidney, periosteum, endosteum and perichondrium, all fix the dye intensely after but a few minutes. Tendon acts in a similar fashion, although the stain is not so deep.

For example, the central tendon of the diaphragm, or the Achilles tendon, binds trypan blue in a diffuse manner, easily visible in both fixed and unfixed preparations, long before there are any granules formed intracellularly.

Reticulin.—Reticulin may be defined as the connective tissue fibers which stain black with ammoniacal silver solutions, in contrast to collagen which stains brown (or rose to rose-purple, if the section is toned with gold). These argyrophile fibers, which have a very wide distribution in the body, differ markedly among themselves in size. They differ as well in their capacity to fix trypan blue. In fat depots, such as in the mesentery, or more especially the pelvis of the kidney, the intercellular connective tissue shows a vivid degree of vital staining. On the other hand, the network of fine argyrophile fibers which surround skeletal muscle fibers remains completely uncolored. The reticulin of the spleen or bone marrow stains but little or not at all. In the spleen some of the scanty reticulin within the follicles may at times assume a blue color, but a similar staining in the reticulin of the pulp has not been observed. There are undoubtedly several unknown factors involved, of which one may be that of sheer mass.

Elastic Fibers.—The staining of elastic fibers in the blood vessels has been adequately treated by previous investigators. A coloration of elastic fibers in the walls of pulmonary alveoli is readily observable, but this is most evident 24 hours after injection. Special note should be given to the capsule and trabeculae of the spleen. These are composed of dense collagen very rich in elastic fibers, and stain with great brilliance (Fig. 2). The coloration, however, is due to fixation of the dye by both collagenous and elastic fibers, and not to either separately.

The fate of the dye which diffusely stains the connective tissues is readily followed. It is slowly given up and at the same time is aggregated into intracellular granules by colloidopexic cells. As the diffuse staining diminishes, the granules in the histiocytes and other cells progressively increase in number. This is most noticeable in adipose tissue. At 10 minutes after the dye is given, the reticulin framework is clearly outlined in blue. At 4 hours, practically all the dye has been aggregated by the histiocytes and very little remains of the diffuse coloration. Collagen, however, holds the dye for a much longer time.

At 4 hours, a few histiocytes contain granules, but the general intensity of the diffuse staining is not much less than at 10 minutes. By 24 hours most of the diffuse coloration has disappeared from the looser types of collagenous tissue, while, correspondingly, intracellular granules have increased enormously in number. Where the connective tissue is dense, as, for example, in the periosteum, perichondrium, central tendon of the diaphragm, basement membrane of the trachea, and the like, the diffuse color persists even for 72 hours. In such regions not only are the histiocytes and fibroblasts filled with granules (these two cell types are, of course, readily distinguishable on the basis of vital staining), but the intercellular substance maintains a pale blue homogeneous tint. The elastic tissue elements in the blood vessels hold the dye for much longer periods, even after it has disappeared from collagen.

Special Instances

A few organs and tissues deserve special comment.

Kidney.—The dye is excreted very rapidly. Within 2 minutes after an intravenous injection a bluish red urine has been seen. The lumina of the kidney tubules, especially the loops of Henle and the collecting tubules, often contain large masses of precipitated dye. In the kidney substance proper, the reticulin framework and the basement membrane of the tubules are outlined in blue after 10 minutes. This disappears at the end of 4 hours. The first granules seen in the kidney are in the proximal convoluted tubules, that is, in parenchymatous cells. The granules appear after an hour and are situated between the nucleus and the free border of the cell. This occurs long before any granules in histiocytes are visible.

Liver.—Granules of trypan blue are seen within Kupffer cells after 10 minutes. At this time the dye is visible as small spherical masses, and only later do the typical crystal-like flecks and granules appear. The capsule and connective tissue septa, vividly blue after 10 minutes, fade rapidly. At 24 hours, there is but little diffuse staining, and at 72 hours none. During this interval, of course, the Kupffer cells and interstitial phagocytes become heavily crammed with granules, while the endothelial cells contain fewer and smaller dye particles. The liver cells of the mouse contain few or no granules, although the dye is very rapidly excreted into the bile.

Spleen.—The spleen forms a marked contrast to the liver. The capsule and trabeculae maintain their diffuse staining, with but little diminution in intensity at the end of 72 hours. In the pulp the reticulin does not stain. Some in the center of the follicles may be colored after 10 minutes to an hour, but this does not

last. Phagocytized granules are surprisingly scarce at 4 hours, and not very prominent even at 24 to 72 hours. In the parenchyma the intracellular granules are situated almost exclusively in the pulp, and are not nearly so vivid as in the other organs.

Blood Vessels.—The veins show a diffuse staining of their entire walls, which for the most part has quite disappeared at 24 hours. In the arteries the internal elastic laminae and the adventitia stand out sharply, even after 10 minutes. The muscle fibers are unstained. In the medium sized arteries some of the fine elastic fibers of the media appear a pale blue. In the aorta the internal elastic lamella stains almost at once, as do the external lamellae and adventitia, but the elastic fibers of the media are colored only after 24 hours. Figs. 5 and 6 show an aorta at 1 hour and 72 hours, respectively. The reason for the long time required to stain the media is not altogether clear. However, the elastic fibers, once stained, hold the dye very tenaciously.

Brain.—The brain tissue proper, which has no intrinsic connective tissue stroma, does not stain with trypan blue.[1] However, in the brain the dura mater, the stroma of the pineal gland, and the choroid plexuses stain at once, just as does connective tissue in other parts of the body. The leptomeninges can be seen diffusely stained only at times, where the trabeculae are concentrated in sufficient mass. Thus, diffuse staining in the adventitia of the larger blood vessels of the pia is quite readily seen, although color could not be detected in the finer reticulin strands. However, the dye must have attained even the finer meshes, for 24 to 48 hours after a single intravenous injection there are myriads of fine granules within the cells of the pia-arachnoid. Obviously, dye must have been present in order that granules be formed.

The cerebral blood vessels present an unusual feature (Fig. 4). Whereas the internal elastic membrane of other arteries is distinctly colored after 10 minutes, in the arteries of the brain such coloration is not very pronounced until 24 hours have elapsed. Then the elastic membranes appear very distinctly, even after a small dose of dye. The best method of demonstration is as follows: 24 to 72 hours after a single intravenous injection, the mouse is killed, the head perfused with saline through the aorta, the entire brain fixed and cleared by the method of Spalteholz, and examined under the low power of the microscope. The major arteries are clearly outlined by the elastic membranes, and can be followed for considerable distances. Adequate preparations can be made after an interval of 1 hour, but the results are more satisfactory after 24 hours. At the same time the wealth of granules in the pia can be seen to good advantage.

The dye passes through the vascular endothelium with ease, as evidenced by the granular vital staining of the leptomeninges. Why the elastic fibers are late in staining is not clear at present.

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[1] Certain exceptions to this statement, irrelevant here, are discussed elsewhere (8).

The problem of the "blood-brain barrier" in relation to vital staining is discussed in a separate communication (8). Attention should be called to the diffuse vital staining of the infundibular region which is considered more fully in a previous paper (9).

The union of trypan blue and connective tissue is not restricted to cases where the dye is given intravenously. In mice (20 to 25 gm.) receiving 5 mg. of the dye subcutaneously, the vital staining of the intercellular connective tissue is quite vivid after 2 hours, but not as sharp as with the intravenous route. The only intrinsic difference between these two modes of administration is in the speed and concentration with which the dye reaches the circulating blood.

There are certain artifacts which appear in the method of examination described above. At times, if fixation is unduly prolonged, certain nuclei will be outlined in blue. The staining affects the chromatin masses, which are as clearly outlined as if hematoxylin had been applied. This is quite different from the true vital staining of pyknotic or degenerated nuclei, so familiar in vital staining of injured areas. At other times the cytoplasm of certain cells will stain, along with the nuclei, as, for example, some striated muscle fibers, or some of the renal epithelium. Such staining is considered artifact for the following reasons: Staining of nuclear chromatin, when present, invariably occurs in the midst of heavy accumulations of trypan blue in the connective tissue, for example, in the periosteum. Again, the fecal contents of the small intestine are often deeply stained with the dye. Sometimes the epithelial cells in contact with this fecal mass will show a staining of both cytoplasm and nucleus, whereas cells more basally placed in the villi are unstained.

One preparation of the kidney, 24 hours after the administration of the dye, was very instructive. Ordinarily at this time there is no diffuse staining in the kidney except in the blood vessels; but the proximal convoluted epithelium is heavily crammed with dye granules, while the nuclei are totally unstained. In this particular preparation, there were practically no dye granules at all, but the epithelial cells, both nuclei and cytoplasm, were colored a distinct and diffuse blue. When animals are not perfused, there is abundant dye in the blood plasma, which appears as irregular blue granular masses in fixed preparations. In sections, the nuclei of the leucocytes are often stained. This type of cellular staining is quite regularly seen in embedded preparations when an unsuitable fixative, such as Carnoy's, is employed.

It seems probable that the bond between trypan blue and the connective tissue is not sufficiently strong to withstand improper or overlong fixation. There is some postmortem solution of the dye and diffusion into adjacent cellular elements which then exhibit a nuclear and cytoplasmic staining. Rapid and suitable fixation is necessary for the preservation of the color in its original site.

DISCUSSION

It seems clear that trypan blue injected into the blood stream is fixed within a very few minutes by the intercellular connective tissue all over the body. It occurs somewhat more slowly when the dye is given subcutaneously. This fixation, or bond of union between dye and tissue elements, is antecedent to any granular storage or colloidopexic cellular action and is the factor responsible for the macroscopic color a few minutes after the dye is administered. The almost immediate diffuse union between dye and connective tissue is observable in fresh organs as well as in those subjected to proper histological fixatives. It is very readily visible with small doses of dye, as, for example, 2.5 mg. for an 18 gm. mouse. Furthermore, the color remains even if the blood vessels are perfused before examination.

With trypan blue, "affinity" between dye and tissue elements is primary, while intracellular storage by histiocytes is secondary. The ability of certain cells to form intracellular granules is, so to speak, an accidental feature of vital staining. Tissues which are rich in histiocytes will show many granules after 24 to 48 hours, tissues poor in histiocytes will show but few; yet the preliminary union between dye and connective tissue is the same in both instances. This bond of union, whatever its nature (perhaps it is an adsorption phenomenon, as Petroff suggests for elastic tissue) is moderately firm. The elastic fibers hold the dye with greatest intensity, reticulin with the least. Dense collagen is closer to elastic tissue, loose collagen to reticulin. As the dye is given up, it is flocculated into granules by colloidopexic cells, such as histiocytes, which happen to be in the neighborhood.

Roman (10), working with a dye derived from atophan (cinchophen), reported results somewhat similar to those described above. He found a light diffuse staining of collagen, and a deeper staining of elastic fibers, as well as a granular storage in the customary loci. Neither the dye, thienyl-quinoline-carbonic acid, which appears to have been impure, nor the results reported in that publication, have received significant attention in the literature on vital staining.

It seems more than coincidence that histiocytes, which are capable of flocculating the trypan blue, occur in the connective tissue which binds the dye in the first place. These cells seem specialized to segregate the dye which has been adsorbed (let us provisionally use this

term) by the intercellular matrix. Since evolutionary specialization could scarcely take place for the purpose of dealing with trypan blue, the following suggestion seems plausible: that a preliminary bond of union with the connective tissue is a general property of noxae which are dealt with by the reticulo-endothelial system. In this sense trypan blue is merely a prototype which happens to be visible.

SUMMARY

Trypan blue injected intravenously is bound almost at once by the intercellular connective tissue elements all over the body,—by collagen, reticulin, and elastic fibers.

This union of dye and tissue elements is the factor responsible for the early macroscopic blue color and is antecedent to cellular colloidopexic action.

Different examples of connective tissue differ among themselves in their ability to hold the dye.

Diffuse staining of elastic fibers noted by previous observers is merely a special case of the general affinity of connective tissue for the dye.

The evidence suggests that the histiocytes are cells specialized to segregate noxae that become diffusely bound to the intercellular connective tissue matrix.

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EXPLANATION OF PLATE 1

All illustrations are unretouched photographs of organs of mice that had received 5 mg. of trypan blue intravenously. Figs. 1, 2, 3, 5, 6 are of vitally stained tissues fixed in Susa, embedded in paraffin, sectioned at 30 microns, and mounted without counterstain.

FIG. 1. Esophagus. Mouse killed after 1 hour. × 73.5.

FIG. 2. Spleen. Mouse killed after 1 hour. Note the vital staining of the central arteries of the follicles. In the pulp are masses of red cells, which appear dark in the photograph, although under the microscope there is no possibility of confusion with the vitally stained elements. × 31.5.

FIG. 3. Anterior abdominal wall, rolled up before fixation. Mouse killed after 1 hour. × 55.

FIG. 4. Base of a brain (entire) cleared by the method of Spaltcholz, showing the basilar artery and branches vitally stained by the trypan blue. The black masses at the sides are refractive artifacts due to air. The enormous number of fine granules in the meshes of the pia-arachnoid are not visible in the photograph. Mouse killed after 48 hours. × 26.

FIGS. 5 and 6. Thoracic aorta. Fig. 5 taken after 1 hour, Fig. 6 after 72 hours. Red blood cells within the lumina may be compared with Fig. 2. × 147.5.

(King: Vital staining of connective tissues)

QUANTITATIVE STUDIES OF BRUCELLA PRECIPITIN SYSTEMS

I. Precipitation of Homologous Antisera by Brucella Endoantigens*

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Quantitative relationships in the precipitation of antibodies by their specific antigens have been studied in the past few years by several groups of workers (1–15). Heidelberger and coworkers (8–15) have developed a quantitative theory of the precipitin reaction and have applied it to the study of various antigen-antibody systems. They have shown that "if the combination of antigen or hapten were considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody, simple equations expressing in several instances the entire course of the precipitin reaction could be derived from the law of mass action."

These equations were found to be of the type

$$\text{antibody N precipitated} = 2RS - \frac{R^2}{A}S^2 \dots\dots\dots\dots(1)$$

in which R is the ratio of antibody nitrogen to antigen nitrogen at a reference point in the equivalence zone, S is the amount of antigen or hapten nitrogen added, and A is the amount of antibody nitrogen precipitated at the reference point (10).

Dividing equation (1) through by S, the equation

$$\frac{\text{antibody N}}{S} \text{ in the precipitate} = 2R - \frac{R^2}{A}S \dots\dots\dots\dots(2)$$

is derived. This is the equation of a straight line obtained by plotting $\frac{\text{antibody N}}{S}$ in the precipitate against the amounts of antigen N added. This equation permits the evaluation of the constants 2R and $\frac{R^2}{A}$ for any given serum.

An empirical relation (11, 12) yielded in some instances an even closer approximation to a straight line than that obtained by (2). The equation of this line is

$$\frac{\text{antibody N}}{S} \text{ precipitated} = 3R'' - 2\sqrt{\frac{(R'')^2(S)}{A}} \quad \ldots\ldots\ldots\ldots (3)$$

Since these relationships were found to apply to each of the several systems studied by Heidelberger and associates, it seemed of interest to extend these studies to antigens from the brucella group of organisms. This was made possible by the preparation of purified antigens, which have been designated endoantigens from the brucella group of organisms (16). These substances are comparable to the "somatic O antigens" which Boivin and Mesrobeanu (17) believe present in all Gram-negative organisms. The endoantigens contain polysaccharide, lipide, and possibly amino acids, are very toxic for normal guinea pigs and precipitate brucella antisera to extreme dilutions. The endoantigens from the three species of the brucella are not identical.

Among the antigen-antibody systems studied by Heidelberger and associates, there were two systems, crystalline egg albumin-egg albumin antibody and crystalline horse serum albumin and its homologous antibody, in which the antigens were colorless nitrogen-containing compounds. Since the brucella endoantigens come within the scope of this class of compounds it was decided to employ their procedure for this type of reaction (12) in the study of brucella antigen-antibody systems.

EXPERIMENTAL

Antisera.—The antisera were prepared by injecting separate goats intravenously with suspensions in saline of one-tenth of an agar slant of a virulent strain of each of three species of brucella. The strains used were from the stock collections maintained at this laboratory.

Endoantigens.—Endoantigens for *Br. abortus*, *Br. suis*, and *Br. melitensis*, subsequently referred to as BcA, BcS, and BcM respectively, used in this experiment were prepared as described in a previous publication (16). The endoantigens were prepared for the precipitation tests as follows: A dilution of 1:500 by dry weight in distilled water was prepared. This was clarified by heating

in flowing steam at pH 8 for 10 minutes. The pH was then adjusted to 7 and the solutions were centrifuged and filtered. The opalescent solutions were then diluted so that 1 ml. of each contained 1 mg. dry weight of endoantigen. Sufficient NaCl was added to make a concentration of 0.85 per cent. The nitrogen content of the endoantigens was 8.56 per cent, 10.19 per cent, and 3.69 per cent for the *abortus*, *suis*, and *melitensis* preparations respectively.

Effect of Temperature and Dilutions on Antibody Precipitated by Endoantigens of Brucella from Their Homologous Antisera.—Since Heidelberger and Kendall noted that the time and temperature of incubation influenced the end-results of the precipitin systems studied and that some specific precipitates are more soluble than others, it seemed advisable to determine the effect of temperature and dilution

TABLE I

Total Nitrogen Precipitated from 1 Ml. Serum by the Amount of Endoantigen Indicated in 1 Ml. Saline, unless Otherwise Indicated

Serum + 0.1 mg. homologous endoantigen	37°C. 2 hrs. 4°C. over-night	4°C. 24 hrs.	4°C. 48 hrs.	37°C. 2 hrs. 4°C. over-night Total volume 10 ml.	Difference per ml. between columns 2 and 5
	mg.	mg.	mg.	mg.	mg.
Br. *melitensis*	0.024	0.018	0.023	0.011	0.0016
Br. *suis*	0.054	0.047	0.052	0.042	0.0015
Br. *abortus*	0.076	0.071	0.075	0.055	0.0026

in the three precipitin systems to be considered. The results of this determination are recorded in Table I.

The precipitation of brucella goat antisera by homologous endoantigens is as complete when carried out at 37°C. for 2 hours followed by overnight at 4°C. as when the precipitation is carried on entirely at the lower temperature for 48 hours. 24 hours at 4°C. does not suffice for complete precipitation. The specific precipitates formed are so little soluble in saline solution that this factor may be ignored in the present studies in which a volume of 2 ml. was maintained throughout.

In Tables II, III, and IV are recorded the data obtained from the addition of increasing amounts of the three endoantigens to 1 ml. of their respective homologous antisera. The total volume in each case

TABLE II
Abortus Endoantigen + Abortus Antiserum

BcA added	BcAN added	BcAN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcAN	Antibody pptd.	Ratio A/BcA	Antibody pptd. Calc. by equation (1)	Calc. by equation (3)	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		mg.	mg.	
0.05	0.0042	Total	0.0448	0.0106	9.66	0.253	5.00	0.164	0.182	Antibody excess
0.10	0.0085	"	0.0767	0.0683	8.03	0.426	4.25	0.318	0.337	" "
0.20	0.0171	"	0.1120	0.0949	5.54	0.593	2.96	0.592	0.600	" "
0.50	0.0428	"	0.2176	0.1748	4.08	1.092	2.18	1.150	1.125	" "
0.80	0.0684	0.0595	0.2628	0.2033	3.41	1.270	1.82	1.300	1.309	Excess BcA
1.00	0.0856	0.0791	0.2912	0.2121	2.68	1.325	1.43	1.273	1.379	" "
1.10	0.0941	0.0815*	0.2780	0.1965	2.41	1.228	1.29			" "
1.20	0.1027	0.0844*	0.2684	0.1840	2.18	1.150	1.16			" "
1.50	0.1284	0.0852	0.2352	0.1500	1.76	0.937	0.941			" "
2.00	0.1712	0.1052	0.2016	0.0964	0.91	0.602	0.49			" "

Equation (1): mg. antibody pptd. $= 3.4 \, (BcA) - 2.20 \, (BcA)^2$; A = 1.313 mg.

Equation (3): $\dfrac{\text{antibody}}{\text{endoantigen}}$ in ppt. $= 4.3 - 2.90 \, \sqrt{BcA}$; BcA = 0.965; A = 1.407 mg.

* Determination of excess antigen not run in duplicate.

TABLE III
Suis Endoantigen + Suis Antiserum

BcS added	BcSN added	BcSN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcSN	Antibody pptd.	Ratio A/BcS	Antibody pptd. Calc. by equation (1)	Calc. by equation (3)	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		mg.	mg.	
0.05	0.0051	Total	0.0443	0.0392	7.68	0.245	4.90	0.239	0.249	Antibody excess
0.10	0.0102	"	0.0793	0.0691	6.77	0.432	4.32	0.431	0.427	" "
0.15	0.0153	"	0.1050	0.0897	5.86	0.560	3.73	0.572	0.559	" "
0.20	0.0203	"	0.1225	0.1022	5.03	0.638	3.19	0.671	0.652	" "
0.30	0.0305	"	0.1465	0.1160	3.80	0.725	2.41	0.720	0.745	Neither antibody nor BcS
0.50	0.0509	0.0346	0.1540	0.1194	3.45	0.746	2.19	0.688	0.751	Excess BcS
0.70	0.0713	0.0692	0.1792	0.1100	1.58	0.687	1.01			" "
1.00	0.1018	0.0958	0.1908	0.0950	0.99	0.593	0.63			" "
1.20	0.1222	0.1030	0.1559	0.0529	0.51	0.331	0.327			" "
1.50	0.1528	0.1278	0.1568	0.0290	0.22	0.181	0.144			" "
2.00	0.2038	0.1358	0.1680	0.0322	0.23	0.201	0.151			" "

Equation (1): mg. antibody pptd. $= 5.27 \, (BcS) - 9.57 \, (BcS)^2$; A = 0.723 mg.

Equation (3): $\dfrac{\text{antibody}}{\text{endoantigen}}$ in ppt. $= 6.73 - 7.75 \, \sqrt{BcS}$; BcS = 0.335; A = 0.751 mg.

was 2 ml.   The precipitations were carried out at 37°C. for 2 hours followed by 24 hours at 4°C.   The precipitates were centrifuged, carefully drained, and washed twice with 1 ml. of ice cold saline with careful rinsing of the tubes and agitation.   A third washing seemed to

TABLE IV

Melitensis Endoantigen + Melitensis Antiserum

BcM added	BcMN added	BcMN pptd.	Total N pptd.	Anti-body N pptd.	Ratio AN/ BcMN	Anti-body pptd.	Ratio A/ BcM	Antibody pptd.		Tests on supernatant
								Calc. by equation (1)	Calc. by equation (3)	
m.f.	m.f.	m.f.	m.f.	m.f.		m.f.		m.f.	m.f.	
0.05	0.0018	Total	0.0124	0.0106	5.88	0.066	1.32	0.064	0.068	Antibody excess
0.10	0.0037	"	0.0242	0.0205	5.54	0.128	1.28	0.125	0.127	" "
0.15	0.0055	"	0.0338	0.0283	5.14	0.175	1.16	0.182	0.180	" "
0.20	0.0073	"	0.0440	0.0367	5.02	0.229	1.14	0.236	0.229	" "
0.30	0.0110	"	0.0630	0.0520	4.72	0.324	1.08	0.333	0.318	" "
0.50	0.0184	0.0125	0.0705	0.0580	4.64	0.362	1.06	0.367	0.349	Excess BcM
0.70	0.0258	0.0228	0.1039	0.0811	3.55	0.506	0.82	0.545	0.505	" "
1.00	0.0369	0.0350	0.1271	0.0921	2.63	0.575	0.607	0.614	0.605	" "
1.20	0.0442	0.0369	0.1296	0.0927	2.51	0.580	0.58	0.611	0.612	" "
1.50	0.0553	0.0435	0.1433	0.0998	2.31	0.623	0.53		0.624	" "
2.00	0.0738	0.0576*	0.1484	0.0908	1.57	0.567	0.37			" "
3.00	0.1106	0.0756	0.1480	0.0724	0.95	0.452	0.22			" "
4.00	0.1476	0.0708	0.0922	0.0214	0.30	0.133	0.067			" "
5.00	0.1844	0.0565†	0.0500	0						" "

Equation (1): mg. antibody pptd. $= 1.325 \,(\text{BcM}) - 0.714\,(\text{BcM})^2$; $A = 0.612$ mg.

Equation (3): $\dfrac{\text{antibody}}{\text{endoantigen}}$ in ppt. $= 1.58 - 0.968\,\sqrt{\text{BcM}}$; $\text{BcM} = 1.184$; $A = 0.624$ mg.

* Determination of excess antigen not run in duplicate.

† Calculated by both methods.

have no influence on the precipitates.   Nitrogen determinations were run by a modification of the micro Kjeldahl method.   The supernatants were tested for the presence of excess antigen and antibody by adding to aliquots a corresponding fraction of antibody and antigen, respectively.   In the regions of excess antigen and in the inhibition

zones the excess antigen was determined by reference of the total N precipitated from the aliquot of the supernatant, to graphs of total nitrogen precipitated plotted against endoantigen nitrogen precipitated in the region of antibody excess. In some instances, where indicated, better results were obtained by calculating excess antigen by the method given by Heidelberger and Kendall in their study of the egg albumin system (12). All determinations were made in duplicate unless otherwise indicated.
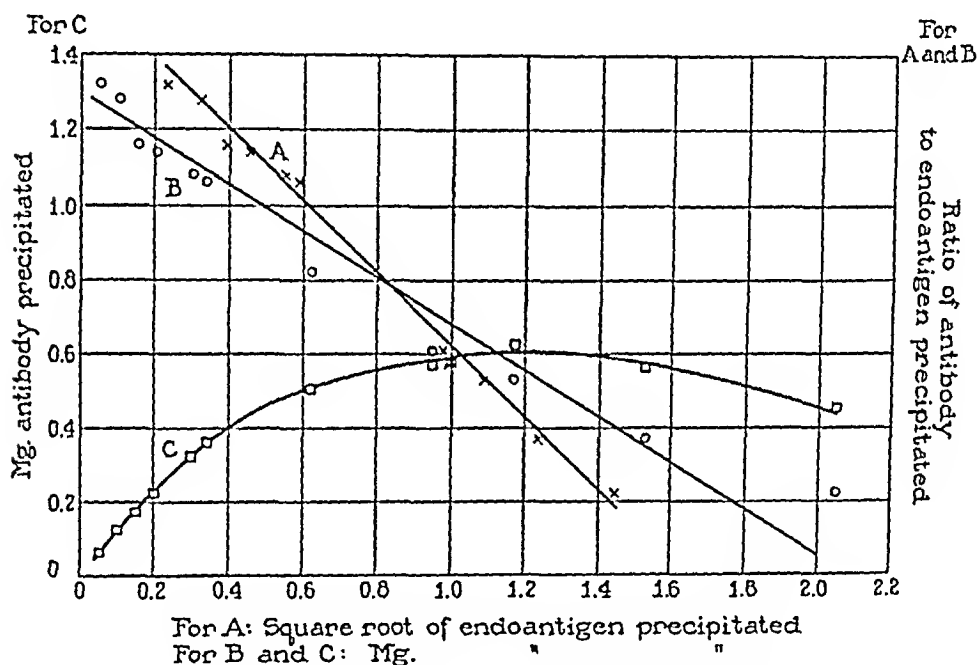
FIG. 1. *Melitensis* serum + *melitensis* endoantigen.

In Fig. 1 are presented the graphs produced from the data in Table IV. The graphs for the two other endoantigens are extremely similar and are not presented.

DISCUSSION

From Tables II, III, and IV and from Fig. 1, it is evident that these three precipitin systems proceed in general, in the same manner as those studied by Heidelberger *et al.* Thus, by plotting the experimental combining ratios of antibody N and endoantigen N against milligrams of endoantigen N precipitated and against the square root

of endoantigen N precipitated, it is possible in each case to derive equations which describe the behavior of the system.

Data obtained in the study of cross precipitations (21), and further data which have not been included in this paper, have shown some suggestion that the nitrogen content of the endoantigen may not always be intimately connected with its ability to precipitate antiserum. For this reason Fig. 1 and equations (1) and (3) for each of the endoantigens have been prepared from substance-combining ratios, rather than N-combining ratios. Although the endoantigens upon careful examination (16) have seemed to be pure substances, this suggestion of occasional lack of correlation necessitates consideration of the present data as provisional, to be corrected by a purity factor if that is found necessary upon further examination of the endoantigens.

If the data for *Brucella abortus* endoantigen be graphed it will be noticed that the initial ratios are much too high to strike the graphs of the linear equations. Yet, if the data be graphed according to equations for systems showing an antibody-antigen combining ratio greater than 2R, agreement with the experimental ratios is entirely lacking. Similar data encountered by Heidelberger *et al.* were believed to show that, whereas the reaction behaved generally according to equation (2), there was a small amount of antibody present capable of reacting with the antigen in ratios greater than 2R. It will be noticed that the values of R and A in equation (1) are located at the beginning of the equivalence zone in the equation developed for the *Br. suis* system, but that these two values are located well within the equivalence zone in the equations developed for *Br. abortus* and *Br. melitensis*.

The data obtained in the inhibition zones gave similar values whether treated by the simple or by the more accurate complex calculation developed by Heidelberger and associates. Since these authors have demonstrated conclusively (11, 12, 15) that the entire amount of dissolved antigen-antibody complex in the supernatant in this zone is precipitable by additional antibody, their method was adopted in this work as perfectly valid.

Since brucella sera are usually calibrated by the determination of agglutinin content, and since agglutinins and precipitins have been shown to be identical (20), the data would imply that the endoantigens

may be used for the more accurate calibration of brucella antisera. For this it would be necessary, as suggested by Heidelberger and Kendall (8), to determine the combining ratios of endoantigen and antibody in a given serum at two, or preferably three points in the region of antibody excess. From these ratios, plotted against the square root of the endoantigen precipitated, an equation of type (3) could be derived, which would permit the calculation of the maximum specific nitrogen of the serum. Conversely, calibrated antisera could be used to detect small quantities of endoantigen.

SUMMARY

It has been shown that the precipitation by the endoantigens of the three species of brucella of their homologous antibodies may be described by equations developed from the law of mass action.

The endoantigens may be used for the accurate calibration of brucella antisera.

The nitrogen-containing constituent of the endoantigens does not always seem to be intimately connected with the ability to precipitate the specific antibodies.

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QUANTITATIVE STUDIES OF BRUCELLA ·PRECIPITIN SYSTEMS

II. The Precipitation of Heterologous Antisera by Brucella Endoantigens*

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The superiority of the quantitative method for the determination of precipitins developed by Heidelberger and Kendall (1) over other methods for determining antigenic differences in closely related bacteria suggests its application to the brucella group as a means of determining differences in the antigens of the three species, namely, *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*.

The antigens used were the endoantigens, the preparation and properties of which have been previously reported (2). It has already been demonstrated (3) that precipitation by the endoantigens of their homologous sera proceeds in a manner which may be described by the equation developed from the law of mass action,

$$\text{mg. antibody pptd.} = 2RS - \frac{R^2}{A^2} S^2 \dots\dots\dots\dots\dots(1)$$

and by the empirical relationship

$$\frac{\text{antibody}}{S} \text{ in ppt.} = 3R'' - 2\sqrt{\frac{(R'')^2 S}{A}} \dots\dots\dots\dots(2)$$

EXPERIMENTAL

Materials.—The antisera and the endoantigens used in this work were those previously described in the study on homologous precipitation. The nitrogen contents of the three endoantigens were 10.19 per cent, 8.56 per cent, and 3.69 per cent for *Br. suis*, *Br. abortus*, and *Br. melitensis* respectively.

TABLE I

Total N Precipitated from 1 Ml. Serum by the Amount of Endoantigen Indicated in 1 Ml. of Saline unless Otherwise Indicated

Serum	0.2 mg. endoantigen	37°C. 2 hrs. 4°C. overnight	4°C. 24 hrs.	4°C. 48 hrs.	Total volume 10 ml. 37°C. 2 hrs. 4°C. overnight	Difference per ml. between columns 3 and 6
		mg.	mg.	mg.	mg.	mg.
Br. melitensis	Br. abortus	0.0784	0.0741	0.0773	0.0584	0.0025
	Br. suis	0.0896	0.0842	0.0871	0.0716	0.0022
Br. suis	Br. abortus	0.0784	0.0736	0.0790	0.053	0.0031
	Br. melitensis	0.0224	0.0198	0.0212	0.005*	0.0058
Br. abortus	Br. suis	0.1232	0.1199	0.1209	0.073	0.0062
	Br. melitensis	0.168	0.1610	0.170	0.128	0.0050

* Total volume 5 ml.

TABLE II

Suis Antiserum + Abortus Endoantigen

BcA added	BcAN added	BcAN pptd.	Total N pptd.	Antibody N pptd	Ratio AN/BcAN	Antibody pptd.	Ratio A/BcA	Antibody pptd. Calc. by Equation (1)	Antibody pptd. Calc. by Equation (2)	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		mg.	mg.	
0.05	0.0042	Total	0.0238	0.0196	4.66	0.122	2.44	0.117	0.127	Excess antibody precipitable by BcA
0.10	0.0085	"	0.0447	0.0362	4.25	0.225	2.26	0.224	0.225	" "
0.15	0.0128	"	0.0634	0.0506	3.95	0.316	2.10	0.312	0.305	" "
0.20	0.0171	"	0.0784	0.0613	3.58	0.383	1.91	0.386	0.369	" "
0.30	0.0256	"	0.1009	0.0753	2.94	0.471	1.57	0.486	0.459	Neither antibody nor endoantigen
0.50	0.0428	"	0.1274	0.0846	1.98	0.528	1.05		0.515	" "
0.70	0.0599	0.0586*	0.1400	0.0814	1.39	0.508	0.743			Excess BcA
1.00	0.0856	0.0784	0.1492	0.0708	0.90	0.443	0.483			" "
1.20	0.1027	0.0970	0.1644	0.0674	0.69	0.421	0.371			" "
1.50	0.1284	0.1224	0.1926	0.0702	0.57	0.438	0.306			" "
2.00	0.1712	0.1652	0.2344	0.0692	0.41	0.432	0.224			" "

Equation (1): mg. A pptd. $= 2.55 (BcA) - 3.10 (BcA)^2$; A $= 0.520$ mg.

Equation (2): $\dfrac{A}{BcA}$ in ppt. $= 3.25 - 3.14 \sqrt{BcA}$; BcA $= 0.476$; A $= 0.519$ mg.

Maximum antibody pptd. by homologous antigen $= 0.751$ mg.

* Duplicate not run.

Effect of Temperature and Dilution on Antibody Precipitated by Endo-antigens of Brucella from Heterologous Brucella Antisera.—The maximum precipitation of nitrogen by a given amount of antigen has been found to occur at different temperatures for different precipitin systems. It has also been found that the precipitates of different precipitin systems have differing solubilities. It was thus necessary to determine these factors for each of the heterologous systems studied. These data are presented in Table I.

TABLE III

Suis Antiserum + Melitensis Endoantigen

BcM added	BcMN added	BcMN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcMN	Antibody pptd.	Ratio A/BcM	Tests on supernatant
mg.	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*		
0.05	0.0018	0	0.0012	0.0012		0.0075		Excess BcM and excess A precipitable by BcM
0.10	0.0036	0	0.0021	0.0021		0.013		" "
0.15	0.0055	0.0006	0.0026	0.0020		0.0125		" "
0.20	0.0073	0.0030	0.0244	0.0214	7.13	0.136	1.68	" "
0.30	0.0110	0.0045	0.0389	0.0344	7.64	0.215	1.76	" "
0.50	0.0184	0.0054	0.0528	0.0474	8.77	0.296	2.036	" "
0.70	0.0258	0.0090	0.0620	0.0530	5.88	0.331	1.35	Excess BcM
1.20	0.0442	0.0306	0.0920	0.0614	2.00	0.384	0.463	" "
1.50	0.0553	0.0483*	0.0769	0.0286	0.59	0.178	0.136	" "
2.00	0.0738	0.0498*	0.0556	0.0058	0.11	0.036	0.027	" "

Equations not applicable.

Maximum antibody pptd. by homologous antigen = 0.751 mg.

* Calculated according to inhibition zone method in (1*d*).

As with homologous brucella precipitin systems the precipitation of antibody by heterologous endoantigens is complete at 37°C. for 2 hours followed by 24 hours at 4°C. In three of the four heterologous systems, as in the homologous systems, the solubility of the precipitates was very slight, and would introduce little error in these studies where the volume was constant at 2 ml. In the other three systems, the precipitation by *melitensis* endoantigen of *suis* and *abortus* antisera and the precipitation by *suis* endoantigen of *abortus* antiserum, the specific precipitates proved slightly more soluble. This solubility factor was considered in the treatment of these systems.

Precipitation of Heterologous Brucella Antisera by the Brucella Endo-antigens.—In Tables II, III, IV, V, VI, and VII are recorded the data obtained by the addition of increasing amounts of the endoantigen designated to 1 ml. of the indicated antiserum.   The total volume was 2 ml. in each case.   The mixtures were incubated at 37°C. for 2 hours followed by 24 hours at 4°C.   The specific precipitates were centrifuged, carefully drained, and washed twice with 1 ml. of ice cold saline with careful rinsing of the tubes and agitation.   Further washing was

TABLE IV

Abortus Antiserum + Melitensis Endoantigen

BcM added	BcMN added	BcMN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/ BcMN	Antibody pptd.	Ratio A/BcM	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		
0.05	0.0018	0.0006	0.0228	0.0222	37.00	0.138	8.53	Excess BcM and excess A precipitable by BcM
0.10	0.0036	0.0018	0.0460	0.0442	24.55	0.276	5.66	" "
0.15	0.0055	0.0037	0.0584	0.0547	14.78	0.341	3.41	" "
0.20	0.0073	0.0043	0.1709	0.1666	38.74	1.041	8.93	Excess BcM
0.25	0.0092	0.0067*†	0.1165	0.1098	16.38	0.686	3.77	" "
0.30	0.0110	0.0080*	0.0948	0.0868	10.85	0.542	2.50	" "
0.50	0.0184	0.0155*	0.0829	0.0674	4.34	0.421	1.01	" "
1.00	0.0369	0.0287*	0.0737	0.0450	1.56	0.281	0.361	" "
1.50	0.0553	0.0400*	0.0696	0.0296	0.74	0.185	0.170	" "
2.00	0.0738	0.0690*	0.0796	0.0106	0.15	0.066	0.035	" "

Equations not applicable.

Maximum antibody pptd. by homologous antigen = 1.407 mg.

* Calculated according to inhibition zone method in (1*d*).

† Duplicate not run.

tried but seemed unnecessary.   Nitrogen was determined by a modified micro Kjeldahl procedure.   The supernatants were tested for the presence of excess antigen and antibody by addition of a corresponding fraction of antibody and antigen respectively to aliquots.   In the regions of excess antigen and in the inhibition zones the excess antigen was usually determined by reference of total nitrogen precipitated from the aliquot of the supernatant, to graphs of total nitrogen precipitated, plotted against endoantigen nitrogen precipitated in the region

TABLE V

Abortus Antiserum + Suis Endoantigen

BcS added	BcSN added	BcSN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcSN	Antibody pptd.	Ratio A/BcS	Antibody pptd. Calc. by Equation (1)	Antibody pptd. Calc. by Equation (2)	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		mg.	mg.	
0.10	0.0102	0.0040	0.0448	0.0108	10.20	0.255	6.49	0.229	0.253	Excess A precipitable by BcS and excess BcS
0.15	0.0152	0.0092	0.0972	0.0880	9.56	0.550	6.09	0.484	0.496	" "
0.20	0.0203	0.0152	0.1232	0.1080	7.10	0.675	4.52	0.714	0.693	" "
0.50	0.0509	0.0260	0.1792	0.1532	5.89	0.957	3.75	0.961	0.911	Excess BcS
0.60	0.0611	0.0448	0.1886	0.1438	3.21	0.898	2.04	0.875	0.918	" "
0.80	0.0815	0.0694	0.2016	0.1322	1.90	0.826	1.21			" "
0.90	0.0917	0.0660*	0.1568	0.0908	1.37	0.567	0.876			" "
1.00	0.1019	0.0938	0.1860	0.0922	0.98	0.576	0.626			" "
1.20	0.1222	0.1084	0.2016	0.0932	0.85	0.582	0.546			" "
1.50	0.1528	0.1412	0.2352	0.0940	0.66	0.587	0.423			" "
2.00	0.2038	0.1898	0.2688	0.0790	0.41	0.493	0.265			" "

Equation (1): mg. A pptd. $= 6.24 \, (BcS) - 9.68 \, (BcS)^2$; A $= 1.005$ mg.

Equation (2): $\dfrac{A}{BcS}$ in ppt. $= 8.32 - 9.40 \sqrt{BcS}$; BcS $= 0.347$; A $= 0.963$ mg.

Maximum antibody pptd. by homologous antigen $= 1.407$ mg.

* Not run in duplicate.

TABLE VI

Melitensis Antiserum + Abortus Endoantigen

BcA added	BcAN added	BcAN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcAN	Antibody pptd.	Ratio A/BcA	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		
0.05	0.0043	Total	0.0448	0.0405	9.41	0.253	5.060	Excess antibody precipitable by BcA
0.10	0.0085	"	0.0560	0.0475	5.58	0.296	2.96	" "
0.15	0.0128	"	0.0672	0.0544	4.25	0.340	2.26	Neither A nor BcA
0.20	0.0171	"	0.0784	0.0613	3.58	0.383	1.91	" " " "
0.50	0.0428	"	0.1008	0.0580	1.35	0.362	0.724	" " " "
0.70	0.0599	0.0560*	0.1124	0.0564	1.00	0.352	0.538	Excess BcA
1.00	0.0856	0.0802	0.1352	0.0550	0.68	0.343	0.366	" "
1.20	0.1027	0.0975	0.1456	0.0481	0.49	0.300	0.263	" "
1.50	0.1285	0.1240	0.1680	0.0440	0.35	0.275	0.189	" "
2.00	0.1712	0.1672	0.2128	0.0456	0.27	0.285	0.144	" "

Equations not applicable.

Maximum antibody pptd. by homologous antigen $= 0.624$ mg.

* Not run in duplicate.

TABLE VII

Melitensis Antiscrum + Suis Endoantigen

BcS added	BcSN added	BcSN pptd.	Total N pptd.	Antibody N pptd.	Ratio AN/BcSN	Antibody pptd.	Ratio A/BcS	Tests on supernatant
mg.	mg.	mg.	mg.	mg.		mg.		
0.05	0.0051	Total	0.0056	0.0005	0.099	0.0031	0.062	Excess antibody precipitable by BcS
0.10	0.0102	"	0.0112	0.0010	0.098	0.006	0.062	" "
0.15	0.0152	"	0.0712	0.0560	3.68	0.350	2.33	" "
0.20	0.0203	"	0.0896	0.0693	3.41	0.433	2.16	" "
0.30	0.0305	"	0.1304	0.0999	3.27	0.624	2.08	Neither A nor BcS
0.50	0.0510	0.0310	0.0784	0.0474	1.52	0.296	0.973	Excess BcS
0.70	0.0713	0.0462	0.0808	0.0346	0.74	0.216	0.476	" "
1.00	0.1020	0.0490	0.0672	0.0182	0.37	0.113	0.236	" "
1.20	0.1222	0.0692	0.0768	0.0076	0.11	0.047	0.069	" "
1.50	0.1528	0.0848	0.0784	0*		0		" "
2.00	0.2038	0.1126	0.0672	0*		0		" "

Equations not applicable.

Maximum antibody pptd. by homologous antigen = 0.624 mg.

* No value obtained by either method of calculation.

TABLE VIII

Serum	Endoantigen	Specific N pptd.	Equations	Precipitation of antibody by initial dilutions of Bc	Precipitation of Bc in initial dilutions	Equivalence zone	Inhibition
		per cent					
Br. suis	Br. abortus	70.5	Applicable	+	+	Extended	Little
	Br. melitensis	51.2	Not applicable	−	−	"	Complete
Br. abortus	Br. suis	68.0	Applicable	+	−	"	Little
	Br. melitensis	73.9	Not applicable	+	−	Narrow	Complete
Br. melitensis	Br. suis	100	" "	−	+	"	"
	Br. abortus	61.6	" "	+	+	Extended	Little

of antibody excess.   In a few instances where indicated the method of calculating excess nitrogen developed by Heidelberger and Kendall in their study of the egg albumin system of (1d) was employed.   All determinations were run in duplicate unless otherwise indicated.

As in the previously considered homologous precipitin systems all figures were transposed to milligrams of antibody precipitated by milligrams of antigen.

DISCUSSION

In Table VIII is presented a résumé of the salient characteristics of the heterologous precipitin systems.

It is evident that there are a variety of types of cross reaction in these precipitin systems, a finding in agreement with Heidelberger and Kendall who found varying types of cross reactions in the heterologous systems which they studied (4a, 4b, 4c). None of the brucella systems, however, conform exactly to any of those studied by these authors.

The similarity of the reciprocal *abortus-suis* systems to each other as well as to the homologous systems (Table VIII, Figs. 1, 2) suggests the "combining units" of the two endoantigens to be nearly identical, in accord with the reported serological similarity of the two organisms. It has been demonstrated by Heidelberger *et al.* that, as evidenced by the declining $\frac{A}{Bc}$ ratio, there is continued combination of antigen with antibody after all antibody has entered into combination; the soluble inhibition zone compound containing one more mole of antigen per mole of antibody than the last insoluble compound. Since in the *abortus* Bc-*suis* antiserum system there appears little specific inhibition, it would seem that there are fewer possible combinations for this system than for the *suis-suis* system. In accord with this, the lower combining ratios of the *abortus* Bc-*suis* antiserum system suggest that the combining unit of *abortus* endoantigen for *suis* antiserum is larger than that for *abortus* antiserum, *i.e.*, that *abortus* endoantigen contains fewer reactive groups for *suis* antiserum than for *abortus* antiserum. The detection of *suis* endoantigen in all dilutions of the *suis* Bc-*abortus* antiserum system and the greater solubility or dissociability of the precipitate in this system suggest a loose combination of antigen and antibody. Inhibition, though occurring in this system, is very gradual, again suggesting a dissociated combination. The combining units of the two endoantigens might be imagined, as suggested by Wilson and Miles (5), as composed of the same components in slightly different proportions. These give rise to a large amount of cross reactive antibody and a small amount of strictly specific antibody.

These systems show some resemblance to the egg albumin-anti-dye system studied by Heidelberger and Kendall (4 *b*).
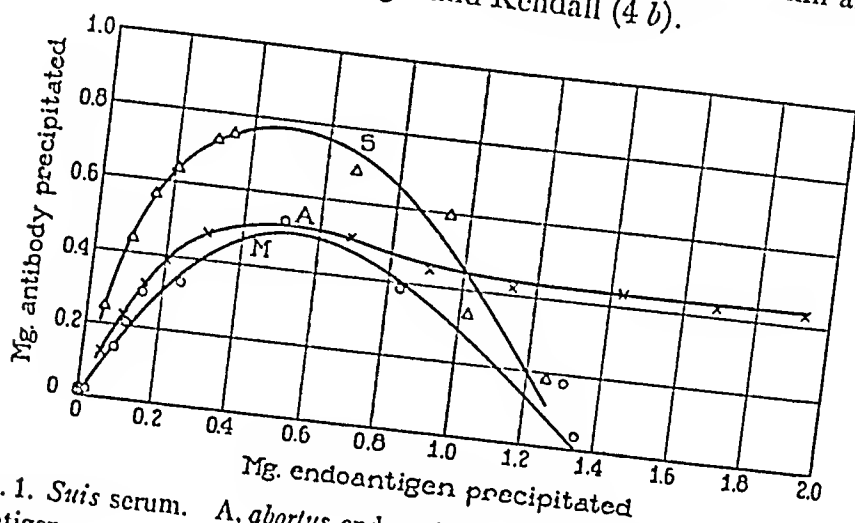
FIG. 1. *Suis* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen.
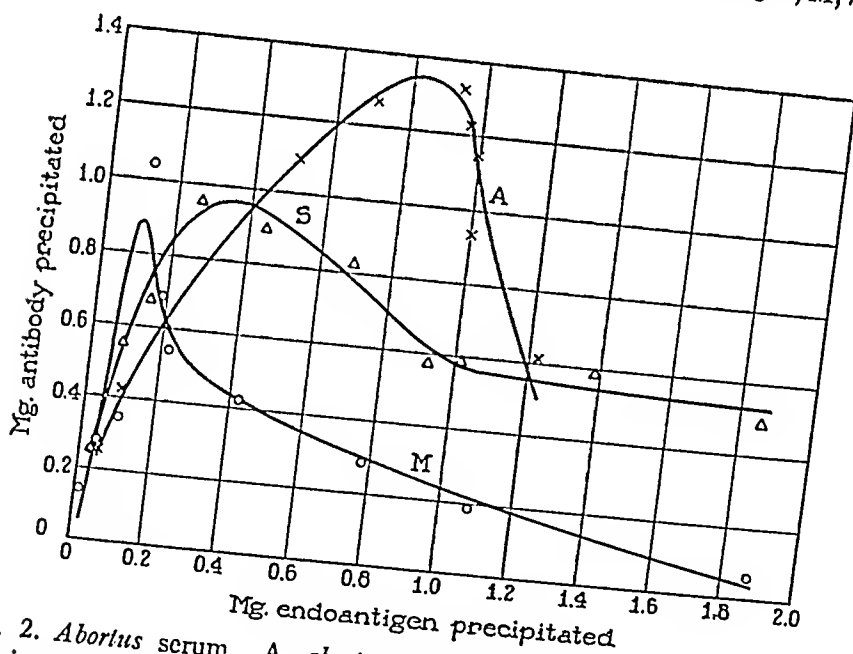
FIG. 2. *Abortus* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen.

he *suis-melitensis* reciprocal reactions resemble each other (Table , Figs. 1, 3), only in the almost complete inhibition and in the

initial concentrations of antigen failing to precipitate antibody. The *melitensis* Bc-*suis* antiserum system appears to have a loosely combined, dissociable precipitate as evidenced by the detection of excess endoantigen at all dilutions, the solubility of the precipitate, and the gradual equivalence and inhibition zones. But half of the antibody in *suis* antiserum is reactive to *melitensis* endoantigen. The lack of antibody precipitation in the initial dilutions is further evidence of the absence of typical *melitensis* antibody in the serum. The *suis* Bc-*melitensis* antiserum system shows unusually abrupt appearance of maxi-
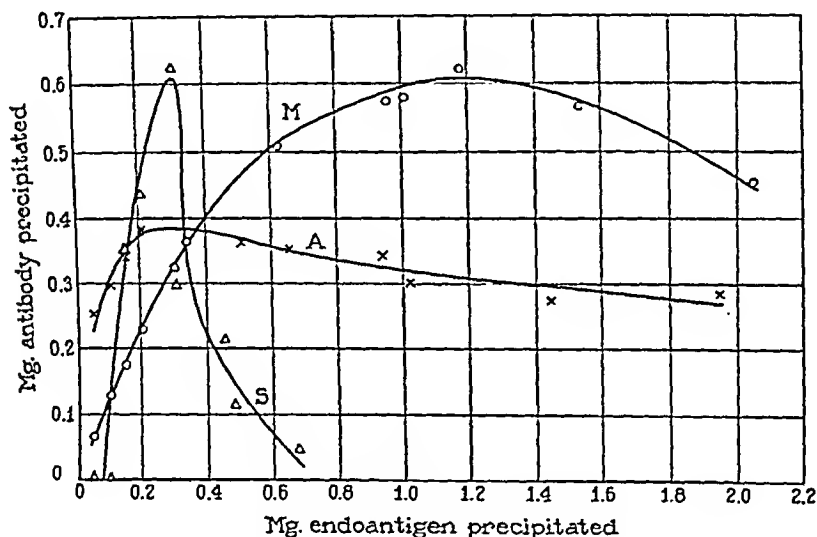
FIG. 3. *Melitensis* serum. A, *abortus* endoantigen; S, *suis* endoantigen; M, *melitensis* endoantigen.

mum precipitation and of inhibition. It can be explained by assuming *melitensis* endoantigen to contain a grouping which occurs much more abundantly in *suis* endoantigen, causing the latter to combine with *melitensis* antibody in relatively small combining units, bringing about precipitation of all the specific nitrogen, and quick formation of the inhibition compound.

The *abortus-melitensis* reciprocal reactions bear little resemblance to each other (Table VIII, Figs. 2, 3). The *melitensis* Bc-*abortus* antiserum reaction is similar to the *suis* Bc-*melitensis* antiserum

system discussed above. It may be explained in an analogous manner, i.e., that *abortus* endoantigen contains a grouping which occurs more abundantly in the *melitensis* compound. It is presumable that, as in the above system, *melitensis* endoantigen can remove all of the specific nitrogen of *abortus* antiserum. The range of maximum precipitation was so brief, however, that this could not be determined experimentally. The *abortus* Bc-*melitensis* antiserum system is very similar to the *abortus* Bc-*suis* antiserum system.

Abortus endoantigen thus reacts similarly to both heterologous sera, whereas *suis* and *melitensis* endoantigens behave quite differently with their heterologous sera.

The interpretation of these cross reactions in terms of the antigenic structure of the brucella is not yet possible as too little is known of the influence of definite chemical combinations on cross precipitation. It is evident, however, that while *suis* and *abortus* endoantigens are very similar, the three endoantigens are serologically distinct, and that these distinctions are displayed in quantitative cross precipitation studies with goat antisera.

To apply these findings to the typing of an organism, it would be necessary after preparing the endoantigen to perform two precipitations in duplicate in the region of antibody excess with a calibrated antiserum, preferably *abortus*. The precipitation of this serum by *melitensis* endoantigen is entirely distinct from the precipitation by the other two endoantigens, while the empirical equation calculated by plotting the antibody precipitated at the two points against the square root of the antigen employed would permit distinction between *Br. suis* and *Br. abortus* endoantigens. The applicability of this procedure using suspensions of whole organisms is being investigated.

In the adaptation of this work to the identification of the infecting organism of an unknown goat antiserum, it would seem probable, from Figs. 1, 2, and 3, that comparison of the precipitates formed by a given amount of the endoantigens would be sufficient. This has not been found to be the case however. Whereas curves of the same types and of the same comparative relationships as those presented in the figures have been produced by the precipitation of other samples of the three brucella goat antisera by the three endoantigens, the curves from different sera are never identical. Thus with some sera all of the

curves are more abrupt, while with others they are more gradual. A given amount of the three endoantigens may thus give varying comparative precipitates with different sera of the same species of brucella. To identify an unknown brucella goat antiserum it is thus necessary to determine two points of the curve in the region of excess antibody with each of the three endoantigens, and from these points, to determine the equation and sketch the respective curves. The application of this procedure to sera other than goat, particularly to human sera is being attempted.

SUMMARY

Quantitative cross precipitation studies with goat antisera show the three endoantigens of the brucella to be serologically distinguishable. Although the endoantigens of *Br. abortus* and *Br. suis* are very similar, they do not react identically, permitting the serological distinction of the two organisms. These differences in cross precipitation may be used to identify an organism of the brucella group or to determine the organism responsible for a brucella antiserum.

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IMMUNIZATION OF GUINEA PIGS AGAINST LYMPHO-CYTIC CHORIOMENINGITIS WITH FORMOLIZED TISSUE VACCINES

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In preliminary experiments, which will not be reported in detail, a considerable degree of immunity was produced in guinea pigs by repeated injections with homologous tissue suspensions, in which the virus of lymphocytic choriomeningitis had been inactivated by the combined action of formalin and room temperature. The immunity was incomplete in the majority of the cases, for the vaccinated guinea pigs showed fever, and sometimes slight symptoms after the test of immunity. Virus could be demonstrated in the circulation during the febrile period.

Vaccines prepared from the consolidated portions of the lungs of guinea pigs killed when moribund gave the most consistent results, while those made from other organs, for example, the brain and liver, often failed to produce immunity. Since the lungs of guinea pigs dying of choriomeningitis often show no macroscopically visible changes, and since vaccines prepared from relatively healthy parts of infected lungs appeared to be inferior, mixtures of several viscera, such as the lungs, brain, liver, spleen, kidneys, and suprarenals, were used for many vaccines, which usually gave good results. Vaccines prepared from infected mouse tissue had very little immunizing power for guinea pigs, even when they were very rich in virus prior to formolization.

The immunization of mice with formolized vaccines usually failed, no matter whether homologous or heterologous vaccines were used. In one experiment, however, a certain degree of immunity was produced in 9 out of 14 mice vaccinated by 3 intramuscular and intra-

peritoneal injections at 10 day intervals with a vaccine prepared from the brains, lungs, livers, spleens, and kidneys of mice very sick from intravenous injection with virus. These 9 mice developed typical tremors and convulsions on the 6th or 7th day after the intracerebral test injection but recovered, while 14 control mice died in typical convulsions.

The experiments to be reported here are concerned chiefly with the cause for the failure of heterologous vaccines to immunize guinea pigs, the immunizing power of homologous vaccines in relation to their virus content prior to formolization, and the mechanism of the immunity produced in guinea pigs by inactive virus in comparison with that which follows an infection (1).

Methods and Materials

Preparation of Vaccines.—The guinea pigs furnishing the tissue for vaccines were killed when very sick or moribund after pad or intracerebral injection with highly virulent guinea pig passage virus (1). The organs used were removed without delay and ground very finely in a tissue grinder. Enough saline was added to make a 20 per cent suspension by weight. In the earlier experiments the suspensions were filtered through several layers of gauze, but later the filtration was omitted because it was found not to be necessary, provided the tissue was ground very finely. In experiments in which the tissue suspensions were titrated a small portion of the suspension was removed for this purpose soon after grinding. To the remainder of the suspension a sufficient amount of neutral formalin was added to make the final concentration of formalin 1 per cent (0.37 per cent formaldehyde) by volume. The vaccines contained in 50, 100, or 200 cc. Erlenmeyer flasks closed with rubber stoppers were vigorously shaken for a few minutes and then kept in a dark place in the laboratory for 2 days. Thereafter they were stored in the refrigerator for the periods of time indicated below in the text. The vaccine remaining after the removal of the amount needed for the first injection was transferred to test tubes and covered with vaseline until used. Shortly before each injection the free formaldehyde in the vaccines was converted into urotropine by adding ammonium hydroxide until a faintly pink color was obtained with phenolphthalein as indicator. The colorimetric tests were carried out with a few drops of vaccine on a white porcelain plate commonly used for pH determinations according to the indicator method. The "neutralized" vaccine was then injected immediately by the routes indicated in the text. The majority of the guinea pigs to be vaccinated received 2 or 3 injections at 10 or 6 day intervals, because it had been found in previous experiments that repeated injections with vaccine usually conferred a stronger immunity than a single injection.

The vaccines were harmless to the animals, except in a few instances in which a

toxic peritonitis resulted from intraperitoneal injections. Intramuscular injections were occasionally followed by a more or less marked induration at the site of inoculation. The formalin in the concentration used appeared to have a strongly bactericidal action in the case of vaccines prepared from diseased lung, which frequently contained bacteria that were harmful when the suspensions were injected without formalin.

That the virus was completely inactivated in the vaccines is indicated by the fact that not one in over 200 guinea pigs injected developed evidence of infection. Furthermore, in many of the earlier experiments one or two extra guinea pigs were given excessively large doses of vaccine (0.2 cc. intracerebrally, 1 cc. in each hind pad, and 5 cc. intraperitoneally) without producing infection by such treatment. These animals were tested for circulating virus at different times after vaccination, with negative results in all cases.

The strongest evidence for the complete inactivation of the virus in the vaccines is the incomplete immunity produced by them in the majority of the guinea pigs, as will be shown later. This low grade of immunity is in sharp contrast to the very solid resistance induced by small amounts of virus even in guinea pigs which failed to show fever after the immunizing injection (1).

Two experiments were performed to determine the effect of the two inactivating factors, formalin and room temperature, on the virus. In both tests the formalin alone was able to inactivate the virus in vaccines stored in the refrigerator for 9 days without previous incubation at room temperature. Storage for 2 days at room temperature without adding formalin to the vaccines inactivated the virus in one test, but not in the other.

Experimental Animals.—The guinea pigs used weighed from 300 to 400 gm. in the majority of the experiments and were obtained from the Institute stock. The mice used for titrating tissue suspensions were 5 to 6 weeks of age and came from the Institute colony. Both the guinea pig and mouse stocks are free from choriomeningitis. Animals used in experiments on choriomeningitis are kept under isolation as a routine, and contact with infected animals is carefully avoided.

Test of Immunity.—A mixture of two highly virulent guinea pig passage strains (1) was used to test the immunity of vaccinated guinea pigs. In the preliminary experiments control guinea pigs occasionally recovered from the severe disease induced by this virus. Its virulence increased, however, on successive passages until it killed all the control animals in 8 to 14 days after inoculation. The standard dose for the tests of immunity was 0.5 cc. of a 1 per cent guinea pig brain suspension injected in equal parts subcutaneously into the hind pads. This amount of suspension as a rule contained about 100,000 minimal infective doses of virus.

Vaccination of Guinea Pigs with Vaccines Prepared from Guinea Pig and Mouse Tissue

To confirm the results of the preliminary experiments, in which mouse tissue vaccines failed to immunize guinea pigs, while homologous vaccines produced immunity, two other series of vaccines of guinea pig or mouse origin were tested.

It proved difficult to obtain from each species vaccines of about equal virus content, even when mixtures of several organs were used. For this reason 10^{-1} and 10^{-2} dilutions of the vaccines were tested for immunizing power besides the undiluted material. Two mouse tissue vaccines (corresponding to guinea pig tissue vaccines 7 and 8, Table I) had to be discarded because their virus content was inferior.

The vaccines with the corresponding numbers in Tables I and II were prepared, titrated, and injected at the same time, and the guinea pigs inoculated with them were tested for immunity with the same materials. Before formolization the tissue suspensions were titrated in guinea pigs by pad injection with 1 cc. of the decimal dilutions in saline of the vaccines, one animal being used for each dilution. The vaccines were stored at room temperature for 2 days and in the refrigerator for 8 days. The guinea pigs to be vaccinated were injected twice at an interval of 10 days with 1 cc. into each thigh and 2 cc. intraperitoneally, making a total of 8 cc. vaccine per guinea pig. Their immunity was tested 2 weeks after the second vaccination.

The summary of the results in Tables I and II shows that the undiluted guinea pig tissue vaccines produced immunity in every case and that some vaccines were also effective in a 10^{-1} dilution, whereas the vaccines prepared from mouse tissue, with one exception, completely failed to immunize, even when their virus content before formolization was very high.

A comparison of the virus content of the guinea pig vaccines before formolization with their immunizing power suggests a lack of parallelism between the two factors. The result obtained with guinea pig tissue vaccine 7 points in the same direction. In this case the tissue suspension was divided into two parts before formolization. One part was used as a whole, while the other part was centrifuged at 1600 to 1700 R.P.M. for 10 minutes. Vaccines were prepared from the whole suspension as well as from the supernatant fluid. In spite of the fact that both materials gave the same titration end-point, the vaccine

TABLE I

Vaccination of Guinea Pigs with Vaccines Prepared from Guinea Pig Tissue

No.	Vaccine Tissue constituents	Titer	Reaction to test of immunity of guinea pigs vaccinated with different amounts of vaccine Dilution in saline of vaccine		
			10^0	10^{-1}	10^{-2}
1	Brain, lung, spleen	$10^{-7}+$*	+		++++
2	" " "	10^{-6}	0	+	++++
3	Brain, lung, liver, spleen	10^{-6}	0 +	++	++++
4	Lung	10^{-6}	+ +	++++	++++
5	Lung, spleen, suprarenal	$10^{-7}+$	+ +	+	++++
6	Lung, liver, spleen, suprarenal	10^{-7}	+ + +	++++	++++
7	Brain, lung, liver, spleen	10^{-7}	+ ++	++ ++++	++++
8	Brain, lung, heart, liver, spleen, kidney, suprarenal:				
	Whole suspension	10^{-6}	+ ++	++++ ++++	++++
	Supernatant of same suspension	10^{-6}	+++ ++++	++++ ++++	++++

In this and the following tables:

0 = no detectable reaction to the test inoculation.

+ = fever, but no other signs of illness.

++ = fever, slight symptoms and a slight loss of weight, followed by rapid recovery.

+++ = fever, severe symptoms with marked loss of weight, followed by slow recovery.

++++ = died of choriomeningitis.

* The + sign indicates that the titration end-point was perhaps not reached at this dilution.

made from the whole suspension had a greater immunizing power than that from the supernatant liquid.

Inhibition of the Immunity by Injecting Formolized Normal Mouse Tissue Together with the Guinea Pig Tissue Vaccines

The following experiments were performed in an attempt to determine the cause for the failure of heterologous vaccines to immunize

TABLE II

Vaccination of Guinea Pigs with Vaccines Prepared from Mouse Tissue

Vaccine			Reaction to test of immunity of guinea pigs vaccinated with different amounts of vaccine	
			Dilution in saline of vaccine	
No.	Tissue constituents	Titer	10⁰	10⁻¹
1	Brain, lung, spleen	10^{-6}	++++	
2	" " "	10^{-6}	++++	
3	Brain, lung, liver, spleen	10^{-6}	++++ ++++	++++
4	Brain, liver, spleen	$10^{-7}+$	++++* ++++	++++
5	Embryos from an infected pregnant mouse	$10^{-7}+$	++ ++++	++++
6	Brain, lung, liver, spleen	10^{-6}	++++ ++++ ++++	

* This animal received 3 injections with vaccine at 10 day intervals and was tested for immunity 14 days after the 3rd injection.

guinea pigs. Laidlaw and Dunkin (2) also failed with dog distemper and attributed the phenomenon to the clogging of the immunity mechanism by heterologous antigens. This appeared to be a reasonable explanation, but direct experimental evidence for it was lacking. It was therefore decided to add formolized normal mouse tissue to the guinea pig tissue vaccines or to inject it simultaneously with them, in order to determine its effect on their immunizing power.

In the experiments of the first series (Table III) the vaccines and the formolized normal mouse tissue suspensions were mixed (2 parts to 1) shortly before inoculation. One group of guinea pigs received the tissue mixture, and another group the vaccine mixed with a corresponding amount of saline. In the experiments of the second series (Table IV) the vaccine and the normal mouse tissue were injected

TABLE III

Inhibition of Immunity by Heterologous Antigens
Vaccines from Guinea Pig Tissue and Formolized Normal Mouse Tissue Mixed before Injection

	Vaccine		Reaction to test of immunity		
No.	Tissue constituents	Dose of mixtures and route of inoculation	Guinea pigs injected with vaccine + saline	Guinea pigs injected with vaccine + formolized normal mouse tissue	Unvaccinated controls
1	Lung	2 × 3 cc. im* (thighs) at 10 day interval	+ + + +	+ + ++ ++++	++++ ++++ ++++ ++++
2	Lung, liver, spleen, suprarenals, several lymph nodes	2 × 3 cc. im (thighs) + 3 cc. ip at 10 day interval	+ + +	+++ ++++ ++++	++++ ++++ ++++
3	Brain, lung, spleen, suprarenals, liver	" "	+ ++	+ ++++	++++ ++++
4	Lung	2 × 3 cc. ip at 6 day interval	+ ++	++++ ++++	++++ ++++
5	Brain	3 × 3 cc. ip at 6 day intervals	+ + ++++	++++ ++++ ++++	++++ ++++ ++++

* im = intramuscularly; ip = intraperitoneally.

either by different routes (vaccine 5) or by the same route but on opposite sides of the body (vaccines 6 to 11). Saline inoculations were omitted.

The vaccines and the corresponding suspensions of normal mouse tissue prepared from the same organs and in the same manner as the vaccines were kept at room temperature for 2 days and in the refrigerator for 7 to 14 days prior to injection. Ammonium hydroxide was added to the mouse tissue suspensions in the same manner as to the vaccines before the materials were mixed, and the mixtures were inoculated a few minutes after mixing. The tissue constituents,

TABLE IV

Inhibition of Immunity by Heterologous Antigens

Vaccines from Guinea Pig Tissue and Formolized Normal Mouse Tissue Injected Simultaneously but by Different Routes

No.	Tissue constituents	Dosage and routes of inoculation	Guinea pigs injected with vaccine alone	Guinea pigs injected with vaccine + formolized normal mouse tissue	Unvaccinated controls
5	Brain	3 × 2 cc. vaccine ip* + 0.5 cc. f.n.m.t.† in each thigh at 6 day intervals	+ + ++++	++++ ++++ ++++	++++ ++++ ++++
6	Lung	2 × 1 cc. im (thigh) + 1 cc. sc (flank) at 10 day interval. Vaccine injected on left side of body, an equal amount of f.n.m.t. on right side	+ + + + ++	+ + + ++ ++++	++++ ++++ ++++ ++++ ++++
7	"	" "	+ + ++	+ + +	++++ ++++ ++++
8	Liver	" "	+ + ++++ ++++	++ +++ ++++ ++++	++++ ++++ ++++ ++++
9	Brain	" "	++ ++ +++ ++++	++ +++ ++++ ++++	++++ ++++ ++++ ++++
10	Lung	" "	+ +	++ ++	++++ ++++
11	"	1 × 1 cc. im (thigh) + 1 cc. im (back) + 1 cc. sc (hind pad). Vaccine injected on left side, f.n.m.t. on right side	+ + + +	+ +++ ++++ ++++	++++ ++++ ++++ ++++

* ip = intraperitoneally; im = intramuscularly; sc = subcutaneously.

† f.n.m.t. = formolized normal mouse tissue.

the dosage, and the routes of inoculation are indicated in the tables. The guinea pigs were tested for immunity in the usual manner 2 to 3 weeks after the last injection of vaccine.

The results presented in Tables III and IV show that the heterologous normal tissue inhibited the immunity in a certain number of cases. The inhibitory effect was generally more marked when the materials were mixed before injection than when they were inoculated simultaneously on opposite sides of the body. In the one comparative experiment (vaccine 5) the same result was obtained with both methods. As in previous tests, lung vaccines proved superior to vaccines prepared from brain or liver.

The Immunizing Power of Guinea Pig Tissue Vaccines in Relation to Their Virus Content before Formolization

Since the results of the experiments recorded in Table I suggested a lack of parallelism between the virus content of vaccines prepared from guinea pig tissues and their immunizing properties, another series of similar experiments was carried out, in which the titration end-points of the suspensions used for vaccines were more accurately determined. In order to eliminate any interference by bacterial antigens, lung vaccines were not used and the tissues selected were free from cultivable organisms.

The tissue suspensions from which the vaccines were made were titrated immediately after grinding by intracerebral injection of mice with 0.05×10^{-4} to 0.05×10^{-7} cc., 3 mice being used for each dilution in saline. The vaccines were formolized as usual and stored for 2 days at room temperature and for 7 days in the refrigerator. Guinea pigs were vaccinated by 3 intraperitoneal injections at 6 day intervals with 2 cc. undiluted or diluted vaccine, as indicated in Table V, and tested for immunity 15 days after the last vaccination.

The results recorded in Table V show that the immunizing power of the vaccines did not parallel their content of active virus prior to formolization. In the first experiment, 3 vaccines prepared from different tissues of the same animals were compared. The vaccines prepared from the liver, heart, and spleen tissue were inferior to the brain vaccine in spite of the fact that the heart and spleen suspensions probably contained as much virus as the brain suspension and the

TABLE V

Immunizing Power of Guinea Pig Tissue Vaccines in Relation to Virus Content Prior to Formolization

Experiment No.	Tissue from which vaccine was prepared*	Stage of disease at which tissue was removed from guinea pig	Titration by ic† mouse injection prior to formolization	Reaction to test of immunity of guinea pigs vaccinated with different amounts of vaccine — Dilution in saline of vaccine		
				10^0	10^{-1}	10^{-2}
1	Liver	12th day after pad injection when moribund	10^{-4} D D D 10^{-5} D D — 10^{-6} D D — 10^{-7} — — —	+ ++ ++	+++ ++++	++++ ++++
	Heart and spleen	" "	10^{-4} D D D 10^{-5} D — — 10^{-6} D — — 10^{-7} — — —	+ ++++ ++++	++++ ++++	++++ ++++
	Brain	" "	10^{-4} D D D 10^{-5} D D D 10^{-6} — — — 10^{-7} — — —	+ + +	++ ++++	++++ ++++
2	Brain	11th day after pad injection when very sick	10^{-4} D D D 10^{-5} D D D 10^{-6} — — — 10^{-7} — — —	+++ ++++ ++++	++++ ++++	++++ ++++
	"	6th day after ic injection (high fever)	10^{-4} D D D 10^{-5} D D D 10^{-6} D — — 10^{-7} — — —	++ ++++ ?‡	++++ ++++	++++ ++++
3	Brain	9th day after ic injection when moribund	10^{-4} D D D 10^{-5} D D — 10^{-6} — — — 10^{-7} — — —	+ + +	++++ ++++	++++ ++++
	"	4th day after ic injection (moderate fever)	10^{-4} D D D 10^{-5} D D D 10^{-6} D D — 10^{-7} — — —	++ ++++ ++++	++++ ++++	++++ ++++

* Each vaccine was prepared from respective organs of 2 guinea pigs.

† ic = intracerebrally.

D = mouse died, or was killed in typical convulsions.

‡ This guinea pig died from toxic peritonitis 1 day after the 1st vaccine injection.

liver suspension contained more. Since the results obtained with these three vaccines may not be strictly comparable because of their different tissue constituents, the vaccines in the following experiments were all prepared from brain. A comparison of their immunizing properties shows clearly that they did not correspond to the virus content of the suspensions. This was particularly striking in the 3rd experiment, in which the vaccine prepared from brain removed during the earlier stage of the disease was richer in virus but poorer in immunizing substance than the vaccine made from brain obtained from moribund animals.

Experiments Bearing on the Mechanism of the Immunity Induced by Formolized Tissue Vaccines

The following experiments were undertaken in an attempt to determine first, whether prolonged treatment with large amounts of homologous vaccine would lead to a high degree of immunity and, second, whether protective serum antibodies could be produced by such treatment. In previous tests the sera of guinea pigs vaccinated in the usual manner (by 2 or 3 vaccine injections) failed to have any neutralizing effect on the virus, although the guinea pigs were immune.

Sera drawn on the 10th day after the test of immunity as a rule had protective properties. In 2 out of 10 such sera virus and antibodies were present at the same time, that is, the sera were virulent on intracerebral inoculation but failed to infect, and even neutralized about 10 M.I.D. of virus added to them, when injected into the pads. This rather rapid appearance of the antibodies is in contrast to the relatively slow formation of antivirus in unvaccinated guinea pigs after injection with virus (1). Their serum usually contained no demonstrable antibody on the 10th day after inoculation. It is possible, therefore, that the vaccination causes the production of a very small amount of antibody not demonstrable by the usual neutralization test, and that the antibody production is enhanced by inoculation with active virus.

The vaccines used for the "hyperimmunization" of guinea pigs were prepared from the lungs, liver, brain, heart, spleen, kidneys, or suprarenals of guinea pigs moribund after pad injection with virus. The suprarenal vaccines were 10 per cent suspensions by weight, while all the other vaccines were 20 per cent suspensions. They were incubated at room temperature for 2 days and then stored in

the refrigerator for 7 to 14 days. Guinea pigs weighing from 500 to 600 gm. were given alternating intraperitoneal, intramuscular, and subcutaneous injections at 5 to 7 day intervals with varying large doses depending on the amount of vaccine available at the time of inoculation. The number of injections and the total amount of vaccine given are recorded in Table VI. The immunity was tested as usual on the 17th day after the last vaccination. 2 days before the test of immunity 10 cc. of heart blood were taken from each animal in order to obtain serum for protection tests.

TABLE VI

Hyperimmunization of Guinea Pigs with Vaccines Prepared from Guinea Pig Tissue

Tissue from which vaccine was prepared	Guinea pig No.	Number of injections at 5 to 7 day intervals	Total amount of vaccine given	17 day interval between last vaccine injection and test of immunity	Reaction to test of immunity by pad injection with 10^5 to 10^6 M.I.D. of virus	
					Vaccinated guinea pigs	Unvaccinated controls
			cc.		0	++++
Lung	1	7	32		0	++++
	2	7	30.5		+	++++
Liver	3	7	34.5		0	++++
	4	7	34.5		+	++++
Brain	5	7	39.5		0	++++
Heart	6	7	33.5		+	++++
Spleen	7	7	34		+	++++
Kidney	8	6	33		+	++++
Suprarenal	9	6	29.5			

Table VI shows that 4 of the 9 guinea pigs vaccinated became so highly immune that they showed no reaction to the test inoculation which was fatal in all 9 controls. The degree of immunity in these 4 animals, therefore, compares favorably with that of guinea pigs that have gone through an infection (1).

The remaining guinea pigs showed a moderate fever which was of shorter duration than in guinea pigs vaccinated in the ordinary manner. There was practically no loss of weight or condition in all the animals. Some of the guinea pigs that showed fever carried traces of virus in the blood during the febrile

period, while in the 4 guinea pigs without reaction no circulating virus was detected on the 6th and 8th days after the test of immunity. The tests for virus were made by injecting normal guinea pigs intracerebrally with 0.25 cc. heart blood of the vaccinated animals.

The sera obtained from the 9 guinea pigs were pooled, and the mixture was tested for protective antibodies by neutralization and protection tests in guinea pigs. In the neutralization tests the mixtures of serum (0.25 cc.) and virus (0.25 cc.) incubated for ½ hour at room temperature were injected into the pads of guinea pigs as described before (3). The results of the tests presented in Table VII show that the immune serum neutralized approximately one minimal lethal dose (M.L.D.) of virus.

TABLE VII

Neutralization Tests with Serum from Guinea Pigs Hyperimmunized with Formolized Vaccines

Test No.	Serum from	Results of pad injections in guinea pigs with mixtures of serum and virus				
		Dilutions of supernatant of virulent 10 per cent guinea pig brain suspension				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	9 vaccinated guinea pigs	D*	D	S	S	S
	Normal guinea pigs	D	D	D	S	S
2	9 vaccinated guinea pigs		D	D	S	S
	Normal guinea pigs		D	D	D	S

* D = the guinea pig died of choriomeningitis.

S = the guinea pig survived, showed no signs of illness, and was not immune to reinoculation with virus.

The first protection test in which 2 guinea pigs weighing 450 to 500 gm. received 1 cc. of the pooled immune serum intravenously ½ hour before pad injection with 1 to 10 M.L.D. of virus gave a negative result. The test was therefore repeated using larger amounts of serum. A guinea pig was given 3 cc. serum intravenously ½ hour before a similar virus inoculation, and 4 cc. by the same route 5 days later. The animal developed comparatively mild, non-fatal choriomeningitis, while the 2 control animals not treated with serum died. In the next experiment still larger amounts of serum were used. Two guinea pigs received 5 cc. immune serum intravenously 2 hours before pad inoculation with about 10^5 M.L.D. of virus, 5 cc. serum intravenously on the 5th day, and 4 cc. intracardially on the 8th day after the virus inoculation. One of these 2 animals showed fever, but no other definite signs of illness, while the other developed mild choriomeningitis followed by quick recovery. The 2 untreated controls died of choriomeningitis.

These tests indicate that the formolized vaccines, when administered in large amounts, can give rise to protective antibodies, whose concentration, however, is rather low in view of the considerable degree of immunity acquired by the donor animals.

DISCUSSION

The vaccines used in the experiments reported are considered to contain completely inactive virus because of the rigorous treatment given to them, the consistent absence of symptoms in vaccinated animals, and the failure to detect circulating virus in vaccinated guinea pigs. It is possible that a lower concentration of formalin would have sufficed to inactivate the virus and permitted the production of more effective vaccines, but in such a case one could not be certain that one was always dealing with a completely inactive agent. The degree of immunity produced by the vaccines used is far inferior to that induced by modified active virus (1), which immunizes guinea pigs solidly even in cases in which they show no detectable reaction to the virus inoculation.

The inhibition of the immunity by adding formolized normal mouse tissue to the guinea pig tissue vaccines supports the suggestion of Laidlaw and Dunkin (2) that the failure of heterologous vaccines to immunize is due to the clogging of the immunity mechanism by heterologous antigens. Since the ingredients were treated alike before mixing, it is unlikely that the decrease in immunizing power in the mixtures was caused by chemical reactions between their constituents, altering the antigenic properties of the vaccines. The fact that the inhibition of the immunity was more pronounced when the materials were mixed before inoculation than when they were injected simultaneously on opposite sides of the body can be explained by assuming that the clogging process is mainly a mechanical phenomenon, which is most marked when the different antigens reach the same receptive tissues at the same time.

The lack of parallelism between the virus content of homologous vaccines prior to formolization and their immunizing power cannot be satisfactorily explained. One might assume either that compounds of the virus, devoid of infectivity but still antigenic, are formed in the

infected tissues, or that non-infectious split products of the virus take part in the immunization.

In view of the high degree of immunity and the protective antibodies produced by prolonged treatment with large doses of formolized vaccine there is at present no good reason to suppose that the mechanism of the immunity induced by inactive virus differs fundamentally from that of the immunity which follows an infection. The differences seem to be quantitative rather than qualitative.

SUMMARY

Guinea pigs can be immunized against lymphocytic choriomeningitis by 2 or 3 injections with formolized vaccines prepared from a variety of infected guinea pig tissues. Vaccines prepared from the consolidated areas of diseased lungs gave the best results. The immunity produced was partial in the majority of the cases, in that the vaccinated animals as a rule showed fever after the test of immunity and virus was present in the circulation during the febrile period.

Vaccines prepared from infected mouse tissue had no, or very little, immunizing power for guinea pigs, even when prior to formolization they contained at least as much virus as guinea pig tissue vaccines. This failure to immunize appears to be due to the interference by heterologous antigens, since the immunity induced by homologous vaccines was often inhibited when formolized normal mouse tissue suspensions treated in the same manner as the guinea pig tissue vaccines were added to the latter before inoculation. The inhibitory effect of the heterologous tissue was less marked when it was not mixed with the vaccine but injected simultaneously on the opposite side of the body.

The immunizing power of homologous vaccines did not parallel their virus content prior to formolization.

A high degree of immunity, characterized by protective antibodies in the serum, was produced in some guinea pigs by prolonged treatment with large doses of homologous vaccine, while sera of guinea pigs vaccinated in the ordinary manner contained no detectable neutralizing antibodies. It is possible, therefore, that the immunity

produced by inactive virus differs only quantitatively from that induced by an infection.

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IMMUNITY OF MICE FOLLOWING SUBCUTANEOUS VACCINATION WITH ST. LOUIS ENCEPHALITIS VIRUS

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Of the few known central nervous system virus infections of man with an immunology that can be studied experimentally in animals, St. Louis encephalitis in mice lends itself most readily to quantitative analysis. This virus infection, unlike poliomyelitis, can be studied in selectively bred mice which can be used in large numbers and in which the majority of disturbing variables can be eliminated or controlled. Already the pathogenesis of the infection *via* the nasal passages has been studied both in mice of high inborn susceptibility (1) and in closely related mice of high inborn resistance (2). The former succumb to an overwhelming infection of the brain; the latter remain well in spite of small, local foci in the brain. The differences in response of these innately susceptible and resistant mice appear to rest largely in a difference in the brain tissue itself, no other organ or tissue, including the blood, being concerned. Regarding this property of brain tissue as the significant factor in inherent resistance, we proceeded to analyze acquired resistance, especially that acquired resistance developed through exposure to the specific agent. The present studies describe the immunity of mice following subcutaneous or intraperitoneal vaccination with virulent St. Louis encephalitis virus.

Materials

St. Louis virus, strain 3, was passed as routine from brain to brain of susceptible mice. When required for tests, the virus-containing brain was removed from a prostrate susceptible mouse 4 to 5 days following an intracerebral injection. The brain, weighing approximately 0.4 gm., was triturated in a mortar, diluted with 3.6 cc. of hormone broth, pH 7.4, to make an approximately 10 per cent suspension, and centrifuged at 1,000 R.P.M. for 5 minutes. The supernatant of the 10 per cent

suspension was removed and further diluted in a tenfold series. Upon intracerebral injection, 0.03 cc. of the 10^{-7} dilution was fatal to 50 per cent or more of tested mice, hence 3×10^{-9} cc. were taken as the intracerebral titre of virus under these conditions. The brain is said to contain, therefore, approximately 10^9 intracerebral lethal doses of virus. Similarly, upon nasal instillation, 0.03 cc. of the 10^{-4} dilution was fatal to 50 per cent or more of tested mice, hence 3×10^{-6} cc. were taken as the intranasal titre.

Mice used in these experiments were the Swiss strain selectively bred for susceptibility to certain central nervous system viruses (1). When tested with St. Louis virus they were consistent and highly uniform in their reactions. When vaccinated with virulent virus given subcutaneously or intraperitoneally, all animals remained well. Consequently, their subsequent immunity was attributed entirely to the direct effect of the vaccine upon each individual with no disturbing possibility that part of the immunity of the batch might be the result merely of a weeding out of the susceptible individuals through the lethal effect of the vaccine.

EXPERIMENTS

Amount of Immunity Developed. Time of Appearance and Duration of Immunity.—Susceptible mice injected subcutaneously with virulent virus develop an immunity against both intracerebral and intranasal test inoculations. This immunity appears in about 4 days, is active against 10^5 intracerebral and 10^3 intranasal test doses within 1 week, endures for 8 weeks at this approximate level, and disappears in 12 to 24 weeks. The following protocol illustrates some of these findings (Table I).

Experiment 1.—Mice were given 0.5 cc. of virus diluted 1 to 1,000 subcutaneously in the groin. At 1, 7, 14, 20, and 24 weeks batches were tested for intracerebral and intranasal immunity, together with control unvaccinated mice of the same age. Table I shows that the intracerebral immunity was active in 1 week against 10^6 lethal doses, in 7 weeks against 10^5, in 14 weeks against 10^2, and that at 20 and 24 weeks the immunity had practically disappeared. Similarly the intranasal immunity was active in 1 week and at 7 weeks against at least 10^2 doses, in 14 weeks against 10 doses, and at 20 and 24 weeks was negligible.

Similar experiments have given comparable results. Some nasal immunity was detected as early as 4 days following vaccination.

Amount of Virulent Virus Required for Immunization.—A subcutaneous injection of at least 500 intracerebral lethal doses of virus is required to immunize a mouse successfully (Table II).[1]

[1] Cox and Olitsky (*J. Exp. Med.*, 1936, **63**, 745) found that approximately this same amount of virulent equine encephalomyelitis virus is necessary to immunize a mouse.

TABLE I

Amount, Time of Appearance, and Duration of Immunity of Mice Following Vaccination with St. Louis Encephalitis Virus

Mice	Intracerebral test. Dilution of virus							Amount of immunity Intracerebral lethal doses	Intranasal test. Dilution of virus					Amount of immunity Intracerebral lethal doses
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-6}	
Controls	—	—	—	6,* 6, 7	6, 7, 7	7, 7, 9	8, 8, 9		—	—	7, 9, 9, 11, 11	—	—	
Vaccinated (1 wk.)	S, S, S	S, S, S	S, S, S	S, S, S	—	—	—	10^6	S, S, S, S	S, S, S, S	S, S, S, S	—	—	10^2+
Controls	8, 9, S	—	—	5, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 7	6, 7, 8, 8		—	7, 7, 7, 8	7, 7, 7, 9	9, S, S	—	
Vaccinated (7 wks.)	—	S, S, S	S, S, S	S, S, S	—	—	—	10^6	S, S, S, S	S, S, S, S	S, S, S, S	—	—	10^2+
Controls	—	—	—	4, 5, 5, 6	7, 7, 8, 9	8, 8, 9, 10, S	S, S, S, S	—	—	—	5, 5, 6, 6, 6	—	—	
Vaccinated (14 wks.)	—	—	6, 7, 7	8, S, S	—	—	—	10^2	7, 8, 8, 10, S	—	—	—	—	10
Controls	—	—	—	—	—	5, 6, 6	6, 6, 7		—	—	9, 12, S	9, 12, S	S, S, S	
Vaccinated (14 wks.)	—	—	—	—	6, 6, 7	5, 9, S	5, 5, S	1	—	10, 12, S	12, 12, S	9, S, S	S	1
Controls	—	—	—	—	—	7, 7	7, 7		—	—	7, 9	9, S	—	
Vaccinated (20 wks.)	—	—	—	—	—	7, S, S	7, S, S	1±	—	—	11, S, S	9, S, S	—	1±

Part of the page labels the lowest vaccinated groups as "(20 wks.)" and "(24 wks.)".

* Day of death of mouse following injection.

S = mouse remained well 30 days.

— = dilution not tested.

Experiment 2.—A single subcutaneous injection of 1.5 cc. of mouse brain virus diluted from 10^{-3} to 10^{-7} was given to batches of Swiss mice. The 10^{-7} dilution contained approximately 50 intracerebral lethal doses, the 10^{-3} dilution, 500,000 doses. 3 weeks later part of the vaccinated mice, together with unvaccinated controls, were given test virus intracerebrally and part intranasally. The smallest intracerebral dose, 10^{-6}, used in the test contained 100 lethal doses, the smallest intranasal dose, 10^{-4}, contained not more than one nasal lethal dose as determined by many titrations. The test was therefore sufficiently sensitive to detect a small amount of immunity. Table II shows that vaccine containing 50 intracerebral lethal doses conferred no immunity, but that vaccine containing 500 or more units did induce the characteristic high immunity.

TABLE II

Amount of St. Louis Encephalitis Virus Required to Immunize Mice

Immunizing dose Number of intracerebral lethal doses	Intracerebral test Dilution of virus		Intranasal test Dilution of virus	
	10^{-4}	10^{-5}	10^{-3}	10^{-4}
None	4,* 5, 5	6, 7, 7	7, 8, 8	9, 9, 10
50	4, 5, 7	5, 5, 6	7, 8, S	9, S, S
500	S, S, S	S, S, S	9, S, S	6, S, S
5,000	6, S, S	S, S, S	S, S, S	S, S, S
50,000	S, S, S	S, S, S	S, S, S	S, S, S
500,000	S, S, S	S, S, S	S, S, S	S, S, S

* Day of death of mouse following injection.

S = mouse remained well.   Discarded after 30 days.

Additional experiments gave comparable results. Moreover, not only fresh mouse brain virus but virus maintained under other conditions behaved in a similar manner. For example, virus grown in mouse embryo brain plus 10 per cent serum-Tyrode culture in our laboratory titrated 10^{-6}. This culture virus diluted to 10^{-3} to contain 1,000 intracerebral lethal doses immunized mice against the virulent mouse brain virus. Greater dilutions, however, containing less than 1,000 doses, did not protect. Again, brain virus kept in the frozen and dried state gradually decreased in titre until at 12 months, certain samples contained no virulent virus, others about 500 intracerebral lethal doses per 0.03 cc. tested, and still others, about 1,000 lethal doses. The immunizing potency of these preparations was tested in mice by administering a single subcutaneous dose of 0.5 cc. containing

in the first instance 1,000 intracerebral lethal doses, in the second instance 500 doses, and in the third instance non-virulent virus. The first preparations immunized the mice well, the second preparations gave both positive and negative results, while the third preparations, containing no virulent virus, failed to immunize.

Multiple doses of virulent virus gave no more prompt, no greater, nor more enduring immunity than a single dose containing about 10,000 intracerebral lethal doses.

Having found that a single subcutaneous or intraperitoneal injection of 1,000 intracerebral lethal doses of virus into susceptible mice immunized them regularly after 4 to 7 days against 10^5 intracerebral or 10^3 intranasal doses and that this immunity persisted 8 weeks and finally disappeared at about 20 weeks, we undertook further analyses of this immune state and of the immunizing process concerned. Our technique was guided by the view expressed recently by Goodpasture that in the voluminous literature on the subject of immunity there is a great deal about phagocytosis and antibodies and complement in general, but no scientific explanation of an acquired resistance to any infectious agent, due mainly, perhaps, to a lack of knowledge of the pathogenesis of the natural disease (3). Consequently we paid special attention to the question of pathogenesis of infection in the immune mouse as compared to pathogenesis of infection in the normal mouse.

Pathogenesis of Infection in Immune Mice.—The outstanding finding to date in these experiments has been that virus given nasally or directly into the brain of immunes does not gain a foothold in the brain tissue.

Experiment 3.—Forty susceptible mice were each given subcutaneously 0.5 cc. of fresh mouse brain virus diluted 1 to 1,000. 12 days later each received 0.03 cc. of fresh virus diluted 1 to 100 into the intact nasal orifices. Five unvaccinated mice were similarly inoculated. 1, 2, 3, 4, 6, 8, 10, 15, 25, 35, and 45 days after the test inoculation, three mice were sacrificed,—one for sections of the brain and two for testing for the presence of virus in the olfactory bulbs and in the brain. The remaining seven mice were observed for 30 days.

The unvaccinated mice receiving the nasal instillation of virus were dead by the 8th day; the seven vaccinated mice inoculated intranasally and set aside for observation remained well, indicating that the batch of forty test animals were immunized against a nasal

dose fatal to 100 per cent of non-vaccinated animals. None of the twenty-two animals sacrificed and tested for the presence of virus gave positive results and none of the eleven examined for lesions showed anything abnormal in the olfactory bulbs or brain. Apparently the virus instilled intranasally into immunized mice did not reach olfactory bulbs or brain in detectable amounts.

Finally, animals similarly immunized and given a test intracerebral injection of at least 1,000 lethal doses of virus disposed of the virus within 4 days and showed lesions related mainly to the puncture wound of the hypodermic needle used for the test injection.

The pathogenesis of infection in immune mice following nasal or intracerebral injection of virus differs sharply, then, from that in non-immune mice. In the immune mouse the test virus fails to survive in the brain; in the non-immune mouse it reaches the olfactory bulbs within a few hours following nasal instillation, spreads and increases rapidly, overwhelming the mouse (1). These differences appear to be due directly to conditions in the brain tissue or associated fluids.

Lack of Antiviral Properties in Blood Serum of Immune Mice.—The fluids of immune mice, of which blood serum is probably the most representative, may theoretically act directly against the virus which has reached its tissue of predilection, namely, the brain and cord, or in some sort of brain tissue combination, or again they may be entirely inert. The first possibility found no support in a large series of tests in which blood serum from immune mice vaccinated as above, when combined with virus and injected directly into the brain of susceptible mice, in no way altered the pathogenesis of the resulting infection. The serum from immune animals neither hastened destruction nor reduced titre of the virus. The following protocol illustrates the equally high titre of virus mixed with sera or with optimum broth diluent (Table III).

Experiment 4.—Susceptible mice were divided into two batches. One received an immunizing dose of fresh mouse brain virus, 0.5 cc. of 1 to 1,000 dilution intraperitoneally; the other remained untreated as controls. After 1 week, part of the vaccinated animals were tested for immunity and part were bled for serum for a neutralization test. Equal parts of serum were mixed with equal parts of mouse brain virus in various dilutions in broth. The mixtures were incubated for 2 hours

at 37°C., left standing for 2 hours at 23°C., and finally, each dilution was injected in 0.03 cc. amounts intracerebrally into four Swiss mice. Virus was also mixed with serum from unvaccinated mice and tested in a similar manner.

Table III shows that the virus was as active when mixed with serum from immune mice as when mixed with serum from unvaccinated mice or with broth alone.

Tests for antiviral substances in the sera of animals vaccinated as above have been made throughout the 10 to 20 week span of immunity (4). During the 4 to 8 week period, when the immunity of the mice

TABLE III

Absence of Neutralizing Antibodies in Sera of Immune Mice Following Vaccination with St. Louis Encephalitis Virus

Virus diluted with	Mice injected with 0.03 cc. mixtures of serum plus virus diluted		
	10^{-5}	10^{-6}	10^{-7}
Broth	5,* 5, 5, 5, 5	5, 5, 5, 7, 7, S	11, S, S, S, S
Serum, normal mouse	5, 5, 5, 6	5, 6, 6, S	7, S, S, S
Serum, vaccinated mouse (1 wk.)	5, 5, 5, 6	6, 6, 9, S	—

Immunity of Mice from Which Test Sera Were Obtained

Mice	Mice injected with 0.03 cc. virus in dilutions					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Normal mice	—	—	—	5, 5, 5, 6	5, 6, 6, S	S, S, S, S
Vaccinated mice (1 wk.)	8, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	—

* Footnotes same as in Table I.

to a test intracerebral or intranasal injection of virus was high, antiviral activity of their sera was not detected; subsequently, however, as the immunity of the mice decreased and finally disappeared, antiviral activity of their sera appeared and increased progressively. In short, not only is antiviral activity of the serum undetectable during the period of maximum immunity of the animal, but it becomes detectable when the active immunity disappears.

Although sera from immune mice do not act against the virus under the above somewhat artificial conditions, it is possible that they may be effective under more natural conditions; this has been

difficult to test. If virus is injected intravenously into immune and non-immune animals, it disappears so promptly that no differences can be detected. Virus injected intraperitoneally, however, into immunes and non-immunes appears in the circulating blood in slightly higher titre and for a slightly longer interval in non-immunes than in immunes. Whether this small difference is attributable to blood or tissues of immune mice is not clear.

Experiment 5.—Fifteen mice were vaccinated with 0.5 cc. of a 10^{-3} dilution of virus given subcutaneously. Fifteen similar mice were left untreated as controls. 1 week later, when the vaccinated mice were known to be immune, they, together with the controls, were given 0.5 cc. of a 0.5×10^{-2} dilution of virus intraperitoneally. At frequent intervals thereafter, vaccinated and control mice were bled from the heart. Undiluted blood and blood diluted 10^{-1} and 10^{-2} were injected in 0.03 cc. amounts intracerebrally into two Swiss mice to titrate the amount of virus present.

The virus content of the blood of immune and non-immune mice is shown in Table IV. Following the intraperitoneal injection of about 2,000,000 intracerebral doses of virus, the blood of both immunes and non-immunes showed about 3,000 doses per cc. within 20 to 30 minutes. This quantity was recovered at intervals during a period of 3 hours and 40 minutes from non-immunes; from immunes the quantity dropped progressively to less than 30 doses per cc. (or 1 per 0.03 cc. which was the limit of sensitivity of the test) at 5 hours. None was recovered from immunes thereafter, although the non-immunes showed 300 doses at 5 hours and 30 at 6 hours.

A search for indirect evidence of activity of serum upon the virus in the animal was made by means of endothelial blockade and splenectomy respectively. Trypan blue was injected intravenously into susceptible mice in various doses ranging from a toxic to a barely coloring level. At different periods thereafter, from 4 to 24 hours, the mice were vaccinated and tested 3 weeks later for their immunity to an intracerebral injection. According to theory, the dye, by blocking the endothelial cells' phagocytic activity, interrupts the development of antibody formation. In these tests, however, despite the injection of dye, the animal developed the usual high grade of intracerebral immunity. Similarly, batches of animals were splenectomized 24 hours before vaccination and 1, 2, and 3 days following vaccination. 3 weeks later their immunity to an intracerebral injection was tested. Again the procedure of splenectomy, calculated to interrupt antibody

formation, did not disturb the development of the standard high level of intracerebral immunity.

At present evidence indicates that the essential difference between the immune and non-immune mouse rests in the changed condition of

TABLE IV

Titre of St. Louis Virus in Blood of Vaccinated and Unvaccinated Mice Following Intraperitoneal Injection

Time after intraperitoneal injection of virus	Source of blood tested	Fate of mice injected with 0.03 cc. blood in dilutions			Titre of virus in blood Lethal intracerebral doses per cc.
		Undilnted	10⁻¹	10⁻²	
10 min.	Vaccinated	—	—	—	—
18 "	Unvaccinated	6,* 6	6, 6		330
24 "	Vaccinated	6, 6	6, 9	6, 13	3,300
28 "	Unvaccinated	6, 6	8, 9	8	3,300
50 "	Vaccinated	6, 6	8		330
50 "	Unvaccinated	4, 4	6, 6	9, 6	3,300+
1 hr. 45 min.	Vaccinated	8, 8			33
1 " 52 "	Unvaccinated	6, 6	6, 8	6, 6	3,300
3 hrs. 38 "	Vaccinated	8	13		33+
3 " 41 "	Unvaccinated	6, 6	6, 8	6	3,300
5 "	Vaccinated				Less than 33
5 "	Unvaccinated	6, 8	9		330
6 "	Vaccinated				Less than 33
6 "	Unvaccinated	6, 8			33
7 "	Vaccinated				Less than 33
7 "	Unvaccinated				" " "
25 "	Vaccinated				" " "
25 "	Unvaccinated		.		" " "
30 "	Vaccinated				" " "
30 "	Unvaccinated				" " "
48 "	Vaccinated				" " "
48 "	Unvaccinated				" " "
72 "	Vaccinated				" " "
72 "	Unvaccinated				" " "

the brain tissue itself, the tissue to which the virus has a special predilection.

Fate of Virus Employed for Vaccine.—The question of how the brain tissue becomes altered is being investigated. Does the virus in the vaccine circulate in the blood, penetrate the brain capillaries, and come into intimate contact with brain cells, or does it localize else-

where and produce an immunizing substance which then reaches brain tissue through the circulation? Previous tests (1) showed that virus injected as a vaccine subcutaneously or intraperitoneally was present within 10 minutes in the blood stream in large amounts but that rarely after 1 and never after 5 days was it found in blood or organs except the spleen. Moreover, the mice remained well and showed no brain lesions. Since mice invariably contract a fatal encephalitis if as little as 1/1000 cc. of virus-containing blood is injected directly into their brain, or if it is provided access to brain cells through trauma following a sterile intracerebral needle puncture (1), it is unlikely that the brain cells of vaccinated mice were exposed to the virus in the blood.

Persistence of Virus in the Spleen.—Following disappearance from blood and organs, virus remains in the spleen and increases somewhat in titre (1). Moreover, it can be recovered, although with increasing irregularity, up to but not later than 34 days following injection. Throughout this time the immunity of the mouse is at a maximum; shortly afterwards, however, it commences to decline. There would appear to be some relation, therefore, between duration of virus in the animal body and duration of immunity. Further experiments on this question are in progress.

DISCUSSION

The experiments described above are limited strictly to the immunity which follows vaccination with virus by some artificial route. Only to this extent, therefore, may the immunity produced by St. Louis encephalitis virus be compared with experimental immunity produced by other human viruses having special or exclusive predilection for the central nervous system. St. Louis virus, in the first place, is a relatively powerful immunizing agent, protecting the animal against a subsequent injection of a large amount of virus directly into the brain. Thus the St. Louis-immunized mouse will withstand 100,000 lethal doses given intracerebrally, the Japanese B and rabies-immunized mouse about 1,000 lethal doses, whereas the poliomyelitis-immunized monkey fails to withstand even one lethal dose with regularity. Secondly, the immunity of the St. Louis-vaccinated mouse, like that of the Japanese B and rabies-vaccinated mouse, is not permanent. Immunity to St. Louis virus is maximum for 4 to 6 weeks

and disappears by the 10th to 20th week; immunity to rabies is maximum for about 6 months and disappears after 12 months.   Finally, the duration of immunity of the St. Louis-immunized mouse may have some connection with the persistence of virus in the tissue.   This possible relationship is being studied further.

These studies also bear upon the question of whether immunity to this group of viruses is humoral or tissue in nature.   Recent English reviewers of immunity to virus diseases (5–7) regard all acquired specific immunity as the result of a corresponding circulating antibody.   This view seems to rest largely on the fact that in many infections, immunity, whether produced in nature or by artificial means, is associated with specific circulating antibody not otherwise present and that this antibody acts in one way or another against the specific agent.

Not always does this simple relation hold, however, and notable exceptions have recently been studied in the group of central nervous system virus infections.   For example, the monkey vaccinated with poliomyelitis virus develops neutralizing antibodies but is not regularly immune (8–10); the same is true for the mouse vaccinated subcutaneously with rabies virus (11).   Conversely, the mouse vaccinated with St. Louis virus becomes immune but shows no circulating neutralizing bodies.   Therefore, these two sets of contradictions to the general theory above require consideration.

Bedson attempts to reconcile the data on antibody without immunity (6) by assuming that the poliomyelitis neutralizing bodies in the monkey without immunity are derived from resistant tissues and therefore play no part in resistance.   In Sabin and Olitsky's opinion, however (10), there is no evidence that neutralizing antibodies from the poliomyelitis-vaccinated, non-immune monkey differ from those in the convalescent immune monkey.   A further example not cited by Bedson is found in our report of mice vaccinated with rabies virus (11).   If the mouse is vaccinated subcutaneously it develops high titre neutralizing antibodies but no immunity; if vaccinated intraperitoneally with the same suspension, it develops neutralizing antibodies of similar titre plus immunity.   The circulating neutralizing antibodies in the non-immune have not been distinguished from those in the immune mouse.   Hence, with no reason to believe that anti-

bodies in non-immunes differ from those in immunes, the fact remains that the presence of high titre circulating neutralizing antibodies in an animal does not necessarily render it immune.

With regard to the second contradiction, immunity in the absence of circulating antibodies, Bedson (6) and Burnet (7) postulate a mechanism of local formation and functioning of undetectable antibodies, again without basis on fact. Burnet suggests further that this postulate might be tested in a nasally induced neurotropic virus infection in a host in which infection rate is high but morbidity low. Studies on pathogenesis of such an infection have already been reported (2) with no evidence of local formation or functioning of antibodies conditioning infection or morbidity. Rather the initial degree of innate resistance of the brain tissue appears to condition pathogenesis throughout. Hence with no evidence of local formation or functioning of circulating antibodies, the fact remains that animals may show a high degree of immunity to certain central nervous system virus infections in the absence of detectable circulating antibodies.

The present experiments with St. Louis encephalitis virus in mice have special weight in that they express quantitative relations between a very high degree of immunity and a negligible amount of antibody activity, and over periods of time when both immunity and antibodies are changing. The blood and extracted brain tissue of immune animals fail to protect the brain of susceptibles against one lethal dose of virus, while the brain in the living immune mouse resists a direct injection of 100,000 lethal doses of virus. Moreover, immunity and neutralizing antibody titre vary in opposite directions in that antibody is minimum when immunity is maximum and increases as immunity decreases. Finally, studies on pathogenesis of infection in susceptible mice following nasal or intracerebral inoculation of virus show a prompt spread of virus and lesions in close association with nervous tissue. In immune mice, however, 1,000 to 100,000 lethal doses of virus are blocked and destroyed at once, indicating a highly potent antagonism not found in serum. This blocking of a tissue type of pathogenesis in the immune mouse, together with the failure to detect antibody activity in the sera of immunes, and the quantitative variation of immunity and antibody titre in opposite directions indicate that the immunity of the mouse to St. Louis

virus following vaccination rests largely on the acquired refractory state of the brain tissue itself.[2]

At the moment, therefore, immunity in certain of the central nervous system virus infections is not explainable in terms of a circulating antibody acting directly against the virus.

CONCLUSIONS

1. Susceptible mice injected subcutaneously or intraperitoneally with 15,000 intracerebral lethal doses of St. Louis encephalitis virus develop an immunity in 4 to 7 days to 1,000 to 1,000,000 lethal doses given either intracerebrally or intranasally.

2. This immunity persists 4 to 6 weeks, then decreases gradually and disappears after 8 to 12 weeks.

3. More than 1,000 intracerebral doses of virus given as a vaccine do not materially increase the amount or duration of the immunity; less than 1,000 doses give little or no immunity.

4. Test virus injected intracerebrally into immunized mice induces few lesions and is rapidly destroyed; instilled intranasally, it rarely reaches the olfactory lobes or brain.

5. While immunity is maximum, circulating neutralizing antibodies are not detectable. Moreover, the immunity is not affected by endothelial cell blockade or by splenectomy.

6. A few moments after the immunizing virus is given, it can be recovered from the blood in relatively high concentration. After 24 hours, the blood no longer contains demonstrable virus nor do any organs thus far tested except the spleen. The brain and cord remain entirely normal. The spleen, however, becomes enlarged and harbors virus for as long as 30 days.

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[2] The immunity of mice to St. Louis encephalitis infection following vaccination may or may not be similar to that accompanying convalescence from the disease. The same is true of poliomyelitis immunity in the *Macacus* monkey. Hence analogous findings in the St. Louis-vaccinated immune mouse and the poliomyelitis-convalescent immune monkey are, at the moment, of questionable significance. Attention may merely be directed to the fact that in both the vaccinated mouse and convalescent monkey (10, 12), test virus instilled nasally or injected intracerebrally is promptly destroyed in the absence of detectable antibodies.

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CUTANEOUS INFECTIVITY IN EXPERIMENTAL POLIOMYELITIS

Increased Susceptibility after Neurosurgical Procedures[*]

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Present knowledge of experimental poliomyelitis is founded largely upon experience with a few strains of virus highly adapted to the monkey. Valuable as these data are, it is not unlikely that interesting findings would follow the study of a greater variety of strains and, in particular, fresh strains. For example, a comparison of fresh strains, in spite of certain unavoidable irregularities, indicates that the effective routes of inoculation are not identical for all strains (1). Such variations may be responsible for the reports of infectivity by the gastrointestinal (2) and cutaneous (3–6) routes. Intracutaneous inoculation with certain strains has proven infective with relatively small amounts of virus (4, 5). With one of these strains (3) in hand, the present study was undertaken in an effort to determine the pathways of neurotropic propagation of the virus from the skin to the spinal cord in monkeys.

The experiments were begun on the hypothesis that propagation of virus from an intracutaneous site of inoculation to the spinal cord could be prevented by previous division of appropriate neural connections. The denervations were done by the following methods: (*a*) anterior and posterior rhizotomy; (*b*) production of an isolated skin graft by a two stage flap method; (*c*) complete isolation of a limb from its nerve supply. In addition, a few observations were made upon animals after bilateral olfactory neurectomy.

It was soon evident that these procedures were ineffective in preventing the appearance of typical experimental poliomyelitis. In fact, the susceptibility of the denervated animals to cutaneous infectivity was enhanced over that observed in the normal controls. This became obvious because the cutaneous infectivity of the strain diminished in the controls during the study.

Materials and Methods

Description of Strains.—The Wfd. strain was recovered from cord and medulla of a child dead of bulbar poliomyelitis in Los Angeles, California, in the epidemic of the summer of 1934. The cutaneous infectivity of this strain in its early passages and some of its other properties have been described (1, 3, 7). From the 3rd to the 7th passage this strain showed a high degree of infectivity when inoculated into the skin; 7 of 10 monkeys so inoculated developed the experimental disease. In later passages this property diminished.

Five other established strains, previously compared with the Wfd. strain (1, 7), were used in one experiment, and Experiment 7 was done with the fresh McL. strain.[1] It was obtained as glycerolated human cord in September, 1937, from the epidemic in Toronto, Ontario (8), and was first used here without prior animal passage. The cutaneous infectivity of this strain has been noted (4).

The strains were kept in 50 per cent glycerol and distilled water at 4°C. On the afternoon of use they were freshly prepared as 10 per cent suspensions of spinal cord by grinding with sand and cold saline, but in the last experiment, in September, 1937, the grinding was done with powdered pyrex glass and distilled water. The suspensions were centrifuged at 1000 R.P.M. for 5 minutes. The usual dose was 2 cc. of the cloudy supernatant fluid. Intracutaneously, it was given in 10 piqûres of 0.2 cc. each. Intravenously, 2 to 3 minutes were used for inoculation; when it was given rapidly several animals died before recovering from the anesthesia. Intracerebral inoculations were made into the left frontal lobe. For titrations, tenfold dilutions were made in saline and 0.5 cc. volumes were inoculated intracerebrally. All inoculations were done under full anesthesia (ether or nembutal or dial).

Apparently healthy *Macacus rhesus* monkeys of 2 to 3 kilos were used, but some had tuberculosis. None had been used previously except 4 convalescents, included to test the virus; 3 were convalescent from a poliomyelitic infection induced by the Flexner (7) strain and 1 from a new strain (Rl.). Daily rectal temperatures were recorded for 4 weeks following inoculation, unless death or the development of poliomyelitis terminated the experiment. At autopsy, sec-

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[1] For this material we are indebted to Dr. F. F. Tisdall and Dr. L. N. Silverthorne of the Hospital for Sick Children and the University of Toronto, Toronto, Canada.

tions of medulla and cervical, dorsal, and lumbar cord were taken for histological examination and the rest of the cord was saved in 50 per cent glycerol.

Surgical Procedures.—All operations were carried out under nembutal anesthesia (35 mg. per kilo intraperitoneally).

1. Rhizotomy (Section of Anterior and Posterior Spinal Nerve Roots).—Anterior and posterior rhizotomies were done through a lower thoracic and upper lumbar laminectomy. After opening the dura, the motor and sensory roots, usually of the 7th thoracic to the 1st lumbar segments, inclusive, were divided between the
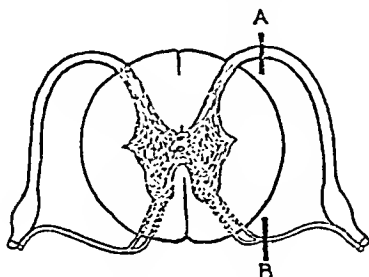
TEXT-FIG. 1. Rhizotomy.   A and B, section of posterior and anterior spinal nerve roots.
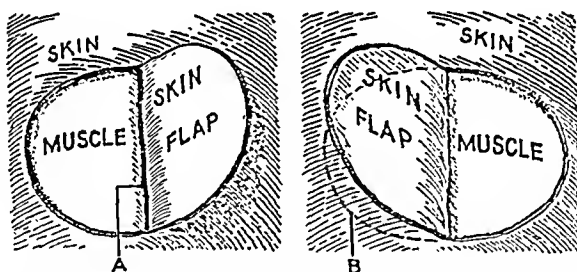
TEXT-FIG. 2. Elevation of skin flap in two stages.   A, first stage showing thread marker.   B, second stage showing line of healed first incision.

spinal cord and the intervertebral foramina, on the right side.   An extradural rhizotomy was done on animal 6-50, Experiment 2, dividing the roots with an electrosurgical cautery.   The dura, muscles, fascia, and skin were then closed in separate layers with silk sutures.   Inoculations were made into the denervated skin of the flank.

2. Skin Flap.—Isolated skin grafts were constructed by a two stage flap procedure.   At the first stage, a flap composed of skin and subcutaneous tissue, was elevated from the underlying muscle in the flank for a distance of about 6 cm., with its pedicle attached anteriorly.   A black silk thread marker was then placed

across the anterior extremity of the undercut area and the flap replaced on its original bed. The U-shaped incision was then closed with silk sutures, but the flap was not anchored to its bed. At the second stage procedure, 9 to 14 days later, an inverted U-shaped incision was made, connecting the open ends of the original U. The skin and subcutaneous tissue were elevated from the underlying muscle until the pedicle of the original flap was completely undercut. The site was readily identified by the black silk marker, inserted at the first stage, the marker being exposed and removed at the second stage. The flap, now attached at its posterior aspect to the original flap, was replaced and sutured as in the first stage procedure. Thus, an area of skin and subcutaneous tissue was completely isolated from the surrounding structures by a two stage procedure. Intra-
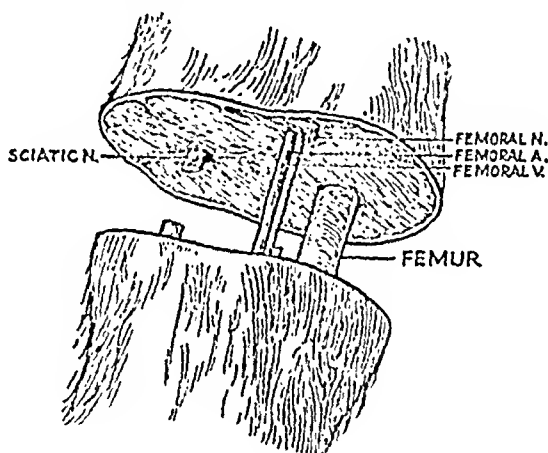
TEXT-FIG. 3. Denervation of limb, showing partial section in mid-thigh.

cutaneous inoculations were then made into the isolated area. The interval between operations was sufficient for establishment of a new blood supply (9), but it is extremely unlikely that nerve regeneration had progressed to any significant extent (10).

3. *Denervation of a Limb.*—This was accomplished by a circular incision around the mid-thigh, entirely dividing the skin, subcutaneous tissue, nerves, muscles, and fascia. Only the femur, femoral artery, and vein were not divided. The artery and vein were stripped completely of all covering, including the adventitia of the artery with its periarterial sympathetic fibers, over a distance of about 1.5 cm. The periosteum was stripped from the femur over a similar distance. The muscles and skin were approximated with silk sutures. Intracutaneous inoculations were then carried out in the denervated area below the knee. The possibility of intact neural communication between the site of inoculation and the cerebrospinal axis would appear remote.

4. *Bilateral Olfactory Neurectomy.*—This was done through a right frontal osteoplastic bone flap. The dura was opened and the frontal lobe elevated,

exposing both olfactory nerves on the floor of the anterior fossa. With the aid of a nerve hook the olfactory bulbs and tracts were separated from the overlying frontal lobes and from 1 to 2.5 cm. of nerve removed. The anterior stump of the bulb was not dislodged from the cribriform plate on the left side. In this manner neural continuity was interrupted without disturbing possible vascular or lymphatic communications.

EXPERIMENTAL

Experiment 1. Cutaneous Inoculation in an Area Denervated by Rhizotomy (Section of Anterior and Posterior Roots of Spinal Nerves).— Following a preliminary experiment, Experiment 1 was devised to

TABLE I

Experiment 1. Cutaneous Inoculation in Denervated and Normal Areas Dec. 22, 1936.

Monkey		Inoculation				Result	
No.	Preparation	Time	Dose	Intracutaneously	Incu-bation period	Paralysis	
		p.m.	*cc.*		*days*		
6-30	None	3.33	2	Face and head	—	Remained well, no paralysis	
6-29	"	3.40	2	Tail	—	" "	
6-27	Rhizotomy right, Dec. 22, 4.30 p.m.	5.50	2	Right flank	6	Severe	
6-28	Rhizotomy right, Dec. 22, 2.15 p.m.	6.03	2	" "	11	"	
6-32	None	6.06	2	" "	—	Fever only	

Virus: 10 per cent No. 4-53; Wfd. strain; generation IX.

compare the infectivity of the virus in denervated and normal areas of skin. The preparatory operations (rhizotomies) were done the day of the inoculations and 3 normal monkeys were included to compare inoculations into skin of head, flank, and tail. Experiment 1 is presented in Table I and the protocols are in the Appendix. The results show that severe poliomyelitis followed intracutaneous inoculations in areas of skin deprived of spinal nerve supply. The ineffectiveness of inoculations into head and tail in Nos. 6-29 and 6-30 and in the flank in 6-32 suggests that the virus had ceased to infect readily in normal skin. It seems, therefore, that the rhizotomies might have

led to an increase in susceptibility of Nos. 6-27 and 6-28. Accordingly, it was decided to collect more data on the effect of denervation by rhizotomy and by other methods.

Experiment 2. Intracutaneous Inoculation after Various Operations.— Skin flaps were elevated in two stages at such intervals that the blood supply would be maintained and yet functional nerve supply would not be reestablished. In other animals olfactory nerves were sectioned; once, this was combined with rhizotomy. The preparatory operations were done during January, 1937, and the inoculations on the 27th of that month. The source of virus was the cord of No. 6-27 from Experiment 1.

The results of Experiment 2 are shown in Table II, and it is obvious that the experience of Experiment 1 was corroborated and extended. In other words, well marked experimental poliomyelitis followed intracutaneous inoculations placed in areas of skin deprived of spinal nerve supply by rhizotomy, or deprived of total nerve supply by elevation of skin flaps. Well marked experimental poliomyelitis followed intracutaneous inoculation in 3 monkeys prepared by sectioning both olfactory nerves. In 2 of these 3 monkeys, postmortem examination revealed that both olfactory nerves had been completely severed. In the third (6-47) a filamentous connection was found between the olfactory bulb and tract on the left side. Most likely this was fibrous tissue but further study was not made to learn its true nature.

The control monkey, No. 6-53, failed to develop experimental poliomyelitis; and 2 others also failed: No. 6-52, sick with tuberculosis, had been prepared by rhizotomy and inoculated in the denervated area, and 6-36, sick with tuberculosis, prepared by rhizotomy and inoculated in the contralateral flank. The comparison of control, No. 6-53, with prepared animals again suggests that the operations might have increased susceptibility to poliomyelitis.

Experiment 3. Intravenous Inoculation; Intracutaneous Inoculation in Denervated Limb; Test of Purity of Virus.—Since the virus had passed sectioned nerves it became desirable to test intravenous inoculations. In the experiment a new type of preparatory operation was included: No. 7-22 was prepared by dividing all structures in the left mid-thigh except femur, femoral artery, and femoral vein, as described

under methods.   The skin and muscles were sewed together and the virus inoculated into the skin of the denervated calf.

Certain animals were included also to test the purity of the virus. This was desirable because the "takes" following denervations raised

TABLE II

Experiment 2.   Intracutaneous Inoculation after Various Operations
Jan. 27, 1937.

Monkey		Inoculation			Incubation period	Result
No.	Preparation	Time	Dose	Route		Paralysis
		p.m.	*cc.*		*days*	
6-40	Skin flap, Jan. 12 and 21	3.25	2	Skin flap	12	Severe*
6-39	" " " " " "	3.28	2	" "	5	Severe
6-50	Extradural rhizotomy right, Jan. 25	3.43	2	Skin right flank	5	"
6-47	Olfactory neurectomy, Jan. 20; rhizotomy right, Jan. 26	3.49	2	" " "	6	Mild
6-54	None, intracerebral control	3.57	0.5	Brain	9	Moderate
6-55	" " "	4.08	0.05	"	11	Severe*
6-44	Olfactory neurectomy, Jan. 15	4.39	2	Skin right flank	7	Severe
6-37	Laminectomy mid-line, Jan. 7	4.49	2	" " "	8	"
6-36	Rhizotomy right, Jan. 6	4.50	2	Skin left flank	—	None*
6-38	Rhizotomy right, Jan. 8	5.05	2	Skin right flank	7	Severe
6-33	Rhizotomy right, Jan. 5	5.09	2	" " "	12	"
6-52	Rhizotomy right, Jan. 27	5.20	2	" " "	—	None. Killed, 24th day*
6-43	Olfactory neurectomy, Jan. 14	5.23	2	" " "	8	Severe
6-53	None, control	5.31	2	" " "	—	Remained well, no paralysis

Virus: 10 per cent No. 6-27 Wfd. strain; generation X; harvested Jan. 4, 1937. (See Experiment 1.)

* Tuberculosis also.

the possibility of accidental contamination of our stock virus.   For these tests 3 convalescent monkeys, paralyzed by 2 heterologous poliomyelitic strains, were inoculated intracerebrally together with

appropriate controls for dosage. 3 rabbits and 2 guinea pigs were inoculated into brain, eye, skin, and peritoneal cavity, and 5 Swiss mice were inoculated intracerebrally. The result of the tests for purity gave no evidence of a contamination. The animals were observed for 4 weeks and 1 of the 3 convalescent monkeys remained well, 1 developed fever and no paralysis, and 1 had fever and slight

TABLE III

Experiment 3. Intravenous Inoculation; Intracutaneous Inoculation in Denervated Limb

Mar. 23, 1937.

Monkey		Inoculation			Result	
No.	Preparation	Time	Dose		Route	Incu-bation period	Paralysis
		p.m.	cc.	per cent		days	
7-04	None	3.44	2	10	Vein	5	Severe*
7-15	"	3.49	2	10	"	11	Mild
7-12	"	3.57	0.5	0.05	Brain	6	Moderate
7-01	"	3.58	0.5	0.05	"	11	"
7-03	"	4.05	2	10	"	4	Severe, purulent meningitis, died
7-14	"	4.08	2	10	"	4	Moderate
7-17	"	4.15	2	10	Skin of flank	—	Remained well, no paralysis
7-06	"	4.17	2	10	" " "	—	" "
7-22	Partial section thigh	4.34	2	10	Skin of calf	11	Severe
7-10	None	4.37	2	10	" " "	4	None, fever only

Virus: 10 per cent No. 6-39; Wfd. strain; generation XI; harvested Feb. 3, 1937. (See Experiment 2.)

* Tuberculosis also.

paralysis. The 3 rabbits and 2 guinea pigs remained well. 4 of the 5 mice remained well and 1 died on the 23rd day. We gave little weight to this mouse because all of 6 Swiss mice survived another intracerebral test with the Wfd. strain. (See Appendix, Experiment A.)

The rest of the experiment is presented in Table III, where it may be seen that the intracerebral infectivity of the virus was considerable. The 2 cc. dose of 10 per cent cord was infective by vein in each of 2

animals, and this dose failed to induce paralysis by the intracutaneous route in all 3 normal monkeys, although 1 of them (7-10) had fever on the 5th to 8th days. However, in No. 7-22, prepared by partial section of the thigh, intracutaneous inoculation in the denervated calf led to severe poliomyelitis. The notes for 7-22 follow.

No. 7-22. *Preparation*: Mar. 23, 1937, 3.00 to 4.30 p.m. Under nembutal anesthesia all the skin, fascia, and muscles were divided in the left mid-thigh down to the femur. The sciatic, femoral, and all other nerves were divided. Only femur, femoral artery, and vein were left intact. All tissue about the femoral artery and vein was stripped clear for about 1.5 cm. The periosteum was divided and scraped from the bone for about 1 cm., completely circumscribing the femur. The muscles were approximated by interrupted mattress suture of silk. The fascia was closed by continuous silk, and a continuous stitch of silk to the skin completed the closure. *Inoculation*: Mar. 23, 4.34 p.m., 2 cc. dose intracutaneously in 10 piqûres in calf of left leg. *Result*: Apr. 3, fever. Apr. 4, tremor, no fever, paralysis of both legs. Apr. 7, prostrate, temperature 95.6°F. Apr. 9, cold, killed. *Autopsy*: Extensive lesions in medulla and moderate lesions in cord. Subcutaneous staphylococcal abscess of site of operation. *Diagnosis*: Severe poliomyelitis.[2]

Experiment 4. Intravenous Inoculation after Olfactory Neurectomy; Intracutaneous Inoculation in Skin Flap; Intracutaneous Inoculation in Denervated Leg; Test of Virus in Convalescent Monkey.—Experiment 4 was planned to repeat some of the previous ones (partial section of thigh and skin flap), and to see if bilateral olfactory neurectomy would prevent infection following intravenous inoculation.

Experiment 4 is shown in Table IV and the protocols are in the Appendix. Two controls, Nos. 7-42 and 7-44, remained well after intravenous inoculation. Thus the intravenous infectivity of the strain appeared to be less than in Experiment 3. A similar decrease in normal intracutaneous infectivity from that originally described (3), was noted in Experiments 1, 2, and 3.

In contrast to the negative results in normal animals (7-42 and 7-44), Nos. 6-69 and 6-81, prepared by bilateral olfactory neurectomy, developed mild poliomyelitis after intravenous inoculation. Severe

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[2] Lesions mean: destruction of ganglion cells, focal accumulations of glial cells, and perivascular cuffing with mononuclear cells.

The estimation of severity of the experimental disease was based on a summation of paralysis, postcritical drop of temperature, and histological findings.

poliomyelitis developed in Nos. 6-97 and 7-21, inoculated in the prepared skin flap, and in 7-45, inoculated intracutaneously in the denervated leg. The immunity of the old convalescent, No. 4-64, is another indication that we were using the virus of poliomyelitis.

The results of intracutaneous and intravenous inoculations in Experiments 1 to 4, together with the results of one preliminary experiment, are summarized in Table V. Of the 19 animals prepared

TABLE IV

Experiment 4. Intravenous Inoculation after Olfactory Neurectomy; Intracutaneous Inoculation in Skin Flap; Intracutaneous Inoculation in Denervated Leg; Test of Virus in Convalescent Monkey

Apr. 14, 1937.

Monkey		Inoculation				Result	
No.	Preparation	Time	Dose		Route	Incubation period	Paralysis
		p.m.	cc.	per cent		days	
6-81	Olfactory neurectomy	3.40	2	10	Vein	4	Mild*
6-69	" "	3.43	2	10	"	5	Mild
7-42	None	3.46	2	10	"	—	Remained well, no paralysis
7-44	"	3.53	2	10	"	—	" "
7-21	Skin flap	4.04	2	10	Skin flap	5	Severe
6-97	" "	4.07	2	10	" "	5	"
7-45	Partial section thigh	4.10	2	10	Skin calf	17	"
7-41	None	4.15	0.5	0.05	Brain	9	Moderate
4-64	Convalescent Flexner 9 mos.	4.17	0.5	0.05	"	—	None

Virus: 10 per cent No. 7-04; Wfd. strain; generation XII; harvested Mar. 30, 1937. (See Experiment 3.)

* Tuberculosis also.

by some form of denervation, 17 developed paralysis after intracutaneous inoculation, while none of the 7 normal controls showed paralysis and only 2 had fever. Accordingly, Experiment 5 was planned to see if denervation by the skin flap method would be effective with other strains of poliomyelitic virus.

Experiment 5. Other Strains of Virus in Skin Flaps.—Skin flaps were elevated in two stages in 10 monkeys and they were used in pairs

for intracutaneous inoculation with 5 other strains (McC.; We.; Flexner; Park; and Aycock) previously compared with the Wfd. strain (1, 7). Fever without paralysis and without lesions in cord or medulla was seen 4 times. A definite positive result was obtained but once; in 1 of 2 monkeys inoculated in the skin flap with the McC. strain (11). This was the first time this strain had been infective by the skin although tests had been made 3 times in previous passages in normal skin. 5 control monkeys inoculated into normal skin with these 5 strains respectively remained well, and the 5 controls inoculated intracerebrally developed poliomyelitis. The scant success just

TABLE V

Summary

Preparation	Total	Paralysis	Fever	Negative
A. Intracutaneous Inoculation				
None..	7		2	5
Laminectomy..............................	1	1		
Rhizotomy.................................	9	7		2
Skin flap.....................................	4	4		
Bilateral olfactory neurectomy..................	2	2		
Bilateral olfactory neurectomy and rhizotomy...	1	1		
Partial section thigh...........................	2	2		
B. Intravenous Inoculation				
None..	4	2		2
Bilateral olfactory neurectomy..................	2	2		

described with the more extended use of the skin flap induced us to simplify the procedure.

Experiment 6. Recovery of Virus (McL.) from Human Cord by Intra-cutaneous Inoculation.—In Experiment 6, 2 monkeys were prepared by elevating the skin flap in one stage, and virus which had not yet been subjected to animal passage was used as the inoculum. The operation consisted of merely the first stage procedure for isolation of an area of skin. The source of virus was a 10 per cent suspension of glycerolated cord from a child (McL.) dead on the 5th day of bulbar poliomyelitis in Toronto, Ontario, in August, 1937. The experiment is shown in Table VI. 5 monkeys were used: 1 for intracerebral and intraperitoneal inoculations and 4 for intracutaneous inoculations. Among the

last, 2 were inoculated into normal skin and 2 into skin flaps. The experimental disease was mild, but it was most marked in No. 8-07, which had a skin flap. From the spinal cord of this monkey the strain was successfully passed to its fourth generation by intracerebral and intraperitoneal inoculations.

It is to be noted that there was a failure in one of the prepared animals, No. 8-06. The explanation for this is not clear, although this skin flap was swollen and fluctuant.

TABLE VI

Experiment 6.   Recovery of Virus (McL.) from Human Cord by Intracutaneous Inoculation in Monkey

Sept. 13, 1937.

Genera-tion	Monkey		Inoculation			Result	
	No.	Preparation	Time	Dose	Route	Incu-bation period	Paralysis
			p.m.	*cc.*		*days*	
I	8-10	None	3.14	2	Skin	13	Mild
	8-09	"	3.23	7	Brain and abdomen	7	Moderate
	8-08	"	3.26	2	Skin	12	Mild
	8-06	Skin flap, Sept. 13	3.40	2	Skin flap	—	Remained well, no poliomyelitis
	8-07	" " " "	3.43	2	" "	7	Moderate
Subsequent Passages							
II	8-07	→ 8-16	Typical poliomyelitis				
III	8-16	→ 8-23	" "				
IV	8-23	→ 8-29	" "				

Virus: Generation I, 10 per cent human cord; later generations, 10 per cent monkey cord inoculated intracerebrally and intraperitoneally.

DISCUSSION

The denervation procedures not only failed to prevent the development of typical experimental poliomyelitis following intracutaneous inoculation into the denervated area, but, in most cases, resulted in an increased susceptibility. Thus, the results must be considered both from the negative and positive aspects. The former relates to the question of strict neurotropism or axonal spread while the latter may be considered in terms of altered neural resistance to the virus.

The various methods of denervation were developed during the course of the experiments in an attempt to establish, if possible, a completely denervated area of skin for intracutaneous inoculation. The failure of the early rhizotomies to prevent infection suggested that the virus might be propagated over the autonomic system from skin to spinal cord. Denervation by means of an isolated area of skin (two stage skin flap) was, therefore, employed. When severe infection occurred with this method, the possibility of autonomic fibers, carried into the isolated area by proliferating blood vessels, was considered. The denervated limb experiments were designed to eliminate this factor. A critical analysis of this procedure leaves no doubt that the somatic nerves were divided; the perivascular sympathectomy was as complete as possible, but the chance retention of one or two intact filaments cannot be disproven. However, this point is probably not as important as might appear on preliminary consideration. In the first place, it is extremely unlikely that the perivascular sympathetic fibers at the mid-thigh level have any neural connection with the site of inoculation in the skin of the calf (12). Secondly, it is evident that intact neural communications, if not completely absent, were at least decreased to an infinitesimal fraction of their normal number. Finally, in striking contrast to the severe experimental disease in the denervated animals, the controls, inoculated in the same site, remained well. Further evidence in support of this thesis is found in the results of intravenous inoculations, which demonstrated the possibility of hematogenous transport of virus; so that from any locus with blood supply the virus could readily reach intact nerves.

However, a certain neural pattern of paralyses indicated a considerable neurotropic tendency of the virus. In 10 of 11 instances where the point could be determined in intracutaneous inoculations, the first limb affected was on the side of the inoculation. The simultaneous onset of fever and paralysis in 4 of 6 monkeys, prepared by skin flaps, is reminiscent of Hurst's (14) results with sciatic inoculations. The failure of bilateral olfactory neurectomy to prevent infection after intracutaneous inoculation is not directly contradictory to the findings of Brodie and Elvidge (15) or Schultz and Gebhardt (16), who employed intranasal inoculations. The successful intravenous inoculations after olfactory neurectomy (Experiment 4) are partially at variance with the experience of Lennette and Hudson

(17), using another strain. However, their failure to infect on intravenous inoculation was preceded by an intranasal (perhaps immunizing) test.

The positive factor, enhanced susceptibility after denervation procedures, was a striking, if rather surprising, result in 17 of the 19 animals tested with the Wfd. strain. The interpretation of this phenomenon rests, at present, chiefly upon a theoretical basis. Alteration of neural resistance probably occurs at the site of nerve injury and as far centrally as the corresponding ganglion cells in the cord. Thus, Webster (18) found that rabies may be localized in the medulla by a mere prick of the tongue in mice. In addition, an abnormal neurovascular relationship is certainly present during the early period at the site of nerve section. Peripheral vascular dilatation in the denervated area undoubtedly resulted from all denervation operations (13) except the olfactory neurectomies. This factor may be of importance when considered in the light of the intravenous infectivity of this strain of virus. Finally, certain of the procedures might disturb the blood brain barrier (19).

Another factor was hypothermia which followed some of the operations and which might have been of importance twice. In view of this and the fact that Wolf (20) found that experimental poliomyelitis could be aborted by hyperthermia, one might question the interpretation of Dalldorf, Douglass, and Robinson (21) when they discount the rôle of fever in the sparing action of dog distemper in experimental poliomyelitis.

Other investigators, notably Flexner and Clark (22), Hurst (14), and Toomey (23), have described methods of increasing susceptibility to experimental poliomyelitis. The denervations acted in this rôle. The degree of increased susceptibility may be appreciated from the fact that on several occasions equal or smaller doses of virus led to a more severe disease on intracutaneous than on intracerebral inoculation.

The skin flaps had the obvious advantage of simplicity and were effective in all of 4 trials with the Wfd. strain. However, with the 6 other strains the method was effective only twice in 12 trials, and this raises considerable doubt concerning the general usefulness of the procedure. Nevertheless, the results of the whole series of operations

show that susceptibility to cutaneous infection can be enhanced. This observation is of considerable interest in view of its possible relation to poliomyelitis in man following tonsillectomy (24) and following subcutaneous inoculations of the virus (25).

CONCLUSIONS

1. Bilateral olfactory neurectomy did not prevent experimental poliomyelitis on intravenous or intracutaneous inoculation.

2. Various operative procedures increased the susceptibility of monkeys to infection with experimental poliomyelitis.

APPENDIX

Preliminary Experiments

Experiment A.—Nov. 13, 1936. *Source of virus*: 10 per cent Wfd. No. 5-92, generation X, harvested Oct. 30, 1936.

Monkey 4-92. *Preparation*: May 8, 1936. Laminectomy; anterior and posterior rhizotomy of spinal roots dorsal 8 to lumbar 2, right. *Inoculation*: Nov. 13, 1936, 2.58 p.m., 2 cc. dose intracutaneously in 10 piqûres in skin of normal (left) flank. *Result*: Nov. 23, fever. Nov. 25, hobbles, weakness of four limbs. Nov. 30, better, killed. *Autopsy*: Moderate lesions in medulla and cord. Rhizotomy verified. *Diagnosis*: Mild poliomyelitis.

No. 6-12. *Preparation*: None. *Inoculation*: Nov. 13, 1936, 3.04 p.m., 2 cc. dose intracerebrally and 9.5 cc. dose intraperitoneally. Nov. 19, fresh supply of virus made up and dose repeated, 2 cc. intracerebrally and 14 cc. intraperitoneally. To test the purity of the virus intracranial inoculation of 6 Swiss mice with 0.03 cc. was done. *Result in No. 6-12*: Nov. 21, fever, Nov. 25, weakness of four limbs, Nov. 30, recovering, killed. *Autopsy*: Moderate lesions in medulla, cervical and lumbar levels of cord; dorsal cord negative. *Diagnosis*: Mild poliomyelitis. *Result in Swiss mice*: All remained well 4 weeks and were discarded.

Experiment B.—Dec. 15, 1936. Nembutal anesthesia. *Source of virus*: 10 per cent Wfd. No. 4-53, generation IX, harvested Apr. 11, 1936.

No. 4-85. *Preparation*: Apr. 24, 1936. Laminectomy; anterior and posterior rhizotomy D. 8 to L. 2, right. *Inoculation*: Dec. 15, 2.44 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank supposedly in denervated area. *Result*: Temperature 96–97°F. during incubation period. Dec. 20, fever, Dec. 21, paralysis right arm, Dec. 23, prostrate, temperature 98°F., killed. *Autopsy*: Extensive lesions in medulla and cord. Rhizotomy verified. 2 or 3 piqûres of virus had been placed above denervated zone. *Diagnosis*: Severe poliomyelitis.

No. 6-26. *Preparation*: None. *Inoculation*: Dec. 15, 2.51 p.m., 2 cc. dose intracerebrally and 2 cc. dose intraperitoneally. Dec. 22, 2 cc. dose intracere-

brally repeated. *Result*: Remained well for 4 weeks, discarded. *Diagnosis*: No poliomyelitis.

No. 6-25. *Preparation*: None. *Inoculation*: Dec. 15, 2.54 p.m., 2 cc. dose intracerebrally and 2 cc. dose intraperitoneally. Dec. 22, 2 cc. dose intracerebrally. *Result*: Dec. 21, fever, Dec. 22, paralysis right arm, Dec. 23, prostrate, temperature 96.1°F., killed. *Autopsy*: Extensive lesions in medulla and cord. *Diagnosis*: Severe poliomyelitis.

Experiment 1.—Dec. 22, 1936. Nembutal anesthesia. *Source of virus*: 10 per cent Wfd. No. 4-53, generation IX, harvested Apr. 11, 1936.

No. 6-30. *Preparation:* None. *Inoculation*: Dec. 22, 3.33 p.m., 2 cc. dose intracutaneously in 10 piqûres in face and head. *Result:* Remained well.

No. 6-29. *Preparation*: None. *Inoculation*: Dec. 22, 3.40 p.m., 2 cc. dose intracutaneously in 10 piqûres in tail. *Result*: Remained well.

No. 6-27. *Preparation*: Dec. 22, 4.30 p.m. Laminectomy; anterior and posterior rhizotomy D. 9 to L. 2, right. *Inoculation*: Dec. 22, 5.50 p.m., 2 cc. dose intracutaneously in 10 piqûres in denervated area of right flank. *Result*: Dec. 28, fever. Jan. 3, 1937, limbs weak. Jan. 4, prostrate, temperature 93.1°F., killed. *Autopsy*: Extensive lesions in medulla and cord. Rhizotomy verified. *Diagnosis*: Severe poliomyelitis.

No. 6-28. *Preparation*: Dec. 22, 2.15 p.m. Laminectomy; anterior and posterior rhizotomy D. 9 to L. 2, right. *Inoculation*: Dec. 22, 6.03 p.m., 2 cc. dose intracutaneously in denervated area of right flank. *Result*: Jan. 2, 1937, fever. Jan. 3, tremor. Jan. 4, four limbs weak. Jan. 5, prostrate, temperature 93.5°F., killed. *Autopsy*: Extensive lesions in medulla and cord. Rhizotomy verified. *Diagnosis*: Severe poliomyelitis.

No. 6-32. *Preparation*: None. *Inoculation*: Dec. 22, 6.06 p.m., 2 cc. dose intracutaneously in right flank. *Result*: Dec. 29 to Jan. 4, fever without other signs. Jan. 19, killed. *Autopsy*: No lesions. *Diagnosis*: Questionable abortive poliomyelitis.

Experiment 2.—Nembutal anesthesia. *Source of virus*: 10 per cent Wfd. No. 6-27, generation X, harvested Jan. 4, 1937. (See Experiment 1.)

No. 6-40. *Preparation*: Jan. 12 and 21. Two stage elevation of skin flap in right flank over 8th to 10th ribs. *Inoculation*: Jan. 27, 3.25 p.m., 2 cc. dose intracutaneously in 10 piqûres in the skin flap. *Result*: Feb. 8, fever. Feb. 9, paralysis right arm. Feb. 11, prostrate, 94°F., killed. *Autopsy*: Moderate lesions in medulla and cord. Caseous tubercles in lungs, liver, and spleen. *Diagnosis*: Severe poliomyelitis; tuberculosis.

No. 6-39. *Preparation*: Jan. 12 and 21. Two stage elevation of skin flap in right flank, over 8th to 10th ribs. *Inoculation*: Jan. 27, 3.28 p.m., 2 cc. dose intracutaneously in 10 piqûres in the skin flap. *Result*: Feb. 1, fever, paralysis of right arm and leg, back weak. Feb. 3, prostrate, temperature below 92°F.,

;illed. *Autopsy*: Extensive lesions in medulla and cord. *Diagnosis*: Severe poliomyelitis.

No. 6-50. *Preparation*: Jan. 25, laminectomy; extradural anterior and posterior rhizotomy, D. 7 to L. 1, right. *Inoculation*: Jan. 27, 3.43 p.m., 2 cc. dose intracutaneously in 10 piqûres in skin of denervated area of right flank. Feb. 1, fever ? (temperature has been irregular), paralysis of legs. Feb. 2, four extremities weak. Feb. 3, prostrate, temperature below 92°F. Feb. 4, found dead. *Autopsy*: Extensive lesions in medulla and cord. Rhizotomy verified. *Diagnosis*: Severe poliomyelitis, fatal.

No. 6-47. *Preparation*: Olfactory neurectomy and rhizotomy. Jan. 20, bilateral olfactory neurectomy. Jan. 26, laminectomy, intradural anterior and posterior rhizotomy, D. 7 to L. 1, right. *Inoculation*: Jan. 27, 3.49 p.m., 2 cc. dose intracutaneously in 10 piqûres in denervated area of right flank. *Result*: Irregular fever since first operation. Feb. 2, agitation, tremor. Feb. 3, paralysis of right arm. Feb. 4, prostrate, temperature below 92°F. Feb. 6, found dead. *Autopsy*: Mild lesions in medulla and cervical cord, lumbar cord negative. Antemortem rupture of esophagus with gastric contents in abdominal and thoracic cavities. Rhizotomy verified. One tiny filament of left olfactory nerve intact. *Diagnosis*: Mild poliomyelitis; rupture of esophagus.

Nos. 6-54 and 6-55. *Preparation*: None. Included for intracranial inoculation of 1/4th and 1/40th of intracutaneous dose. No. 6-54. *Inoculation*: Jan. 27, 3.57 p.m., 0.5 cc. dose into left cerebral hemisphere. *Result*: Jan. 30, fever. Feb. 5, agitation and tremor. Feb. 9, paralysis of left leg. Feb. 13, both legs weak, killed, lowest temperature 103°F. *Autopsy*: Mild lesions in medulla, cervical, and dorsal cord; moderate lesions in lumbar cord. *Diagnosis*: Moderate poliomyelitis. No. 6-55. *Inoculation*: Jan. 27, 4.08 p.m., 0.5 cc. dose 1 per cent virus into left cerebral hemisphere. *Result*: Jan. 30, onset of irregular fever. Feb. 7, tremor, paralysis of left face and right arm, weakness of left arm. Feb. 9, prostrate, temperature 98.5°F. Feb. 10, killed. *Autopsy*: Mild lesions in medulla and dorsal cord, extensive lesions in cervical and lumbar cord. Caseous tubercles in lungs, liver, spleen, and mesenteric lymph nodes. *Diagnosis*: Moderate to severe poliomyelitis; tuberculosis.

No. 6-44. *Preparation*: Jan. 15, bilateral olfactory neurectomy. Jan. 17, animal sick, incision infected. Jan. 21, better. *Inoculation*: Jan. 27, 4.39 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. *Result*: Feb. 3, fever. Feb. 4, tremor, ataxia, right facial paralysis. Feb. 5, paralysis of left arm. Feb. 7, prostrate, temperature 96.2°F. Feb. 9, worse, killed. *Autopsy*: Extensive lesions in medulla and cord. Verified bilateral olfactory neurectomy. *Diagnosis*: Severe poliomyelitis.

No. 6-37. *Preparation*: Jan. 7, laminectomy. D. 7 to L. 1, dura opened but roots were not disturbed; incision in mid-line. *Inoculation*: Jan. 27, 4.49 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. *Result*: Feb. 4, fever, agitation, tremor. Feb. 5, paralysis both legs. Feb. 6; prostrate, temperature

101°F. Feb. 11, same, temperature 99.3°F., killed. *Autopsy*: Moderate lesions in medulla, dorsal and lumbar cord; extensive lesions in cervical cord. Verified laminectomy. *Diagnosis*: Severe poliomyelitis.

No. 6-36. *Preparation*: Jan. 6, laminectomy and intradural rhizotomy of anterior and posterior roots, D. 7 to L. 1, right. *Inoculation*: Jan. 27, 4.50 p.m., 2 cc. dose intracutaneously in 10 piqûres in left (contralateral) flank. *Result*: Feb. 8 to 12 and on 22, irregular fever. No paralysis. Feb. 26, sick, killed. *Autopsy*: No lesions in medulla or cord. Many caseous tubercles in lungs, liver, and spleen. Verified rhizotomy. *Diagnosis*: No poliomyelitis; tuberculosis.

No. 6-38. *Preparation*: Jan. 8, laminectomy and intradural anterior and posterior rhizotomy, D. 6 to D. 12, right. *Inoculation*: Jan. 27, 5.05 p.m., 2 cc. dose intracutaneously in 10 piqûres in denervated area of right flank. *Result*: Feb. 3, fever, agitation, tremor, left facial paralysis. Feb. 4, legs weak. Feb. 6, prostrate. Feb. 10, temperature 97°F., killed. *Autopsy*: Moderate lesions in medulla and dorsal cord; extensive lesions in cervical and lumbar cord. Rhizotomy verified. *Diagnosis*: Severe poliomyelitis.

No. 6-33. *Preparation*: Jan. 5. Laminectomy and intradural anterior and posterior rhizotomy, D. 7 to L. 1, right. *Inoculation*: Jan. 27, 5.09 p.m., 2 cc. dose intracutaneously in 10 piqûres in denervated area of right flank. *Result*: Feb. 8, fever, right facial paralysis. Feb. 9, tremor. Feb. 10, paralysis of right leg, weakness of arms and left leg. Feb. 12, prostrate, temperature 101.3°F., killed. *Autopsy*: Extensive lesions in medulla and lumbar cord; moderate lesions in dorsal cord; cervical cord not saved. At site of laminectomy some attachments present at roots thought to have been divided. It was not clear whether the nerve roots had not been completely divided, or whether the attachments were mere fibrous adhesions. *Diagnosis*: Moderate to severe poliomyelitis.

No. 6-52. *Preparation*: Jan. 27, 2 p.m., laminectomy and intradural anterior and posterior rhizotomy, D. 7 to L. 1, right. *Inoculation*: Jan. 27, 5.20 p.m., 2 cc. dose intracutaneously in 10 piqûres in denervated area in right flank. *Result*: Jan. 31 to Feb. 12, fever 104.2–105.5°F. Feb. 19, emaciated, temperature less than 94°F., killed. *Autopsy*: No lesions in cord, extensive generalized caseous tubercles. *Diagnosis*: No poliomyelitis; tuberculosis. Rhizotomy verified.

No. 6-43. *Preparation*: Jan. 14, bilateral olfactory neurectomy. *Inoculation*: Jan. 27, 5.23 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. *Result*: Feb. 4, fever. Feb. 5, left facial paralysis. Feb. 6, right arm weak. Feb. 8, prostrate, temperature 100°F., Feb. 9, worse, temperature 98°F., killed. *Autopsy*: Extensive lesions in medulla and cord; complete section of olfactory nerves verified. *Diagnosis*: Severe poliomyelitis.

No. 6-53. *Preparation*: None. *Inoculation*: Jan. 27, 5.31 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. *Result*: Remained well. Mar. 1, killed for histological examination. *Autopsy*: No lesions in medulla or cord. *Diagnosis*: No poliomyelitis.

Experiment 3.—See text.

Experiment 4.—Apr. 14, 1937. Nembutal anesthesia. *Source of virus*: 10 per cent Wfd. No. 7-04, generation XII, harvested Mar. 30, 1937. (See Table III.)

No. 6-81. *Preparation*: Mar. 19, 1937. Bilateral olfactory neurectomy. *Inoculation*: Apr. 14, 3.40 p.m., 2 cc. dose intravenously. *Result*: Apr. 18, 106°F. Apr. 21, 106.5°F., agitation, tremor. Irregular fever continued to Apr. 29. Weak and sick thereafter. May 7, 103.5°F., killed. *Autopsy*: Mild lesions in medulla and lumbar cord, none in cervical or dorsal cord; caseous tubercles in lungs, spleen, and liver; bilateral olfactory neurectomy verified. *Diagnosis*: (*a*) Tuberculosis; (*b*) mild poliomyelitis.

No. 6-69. *Preparation*: Feb. 15, 1937, bilateral olfactory neurectomy. *Inoculation*: Apr. 14, 3.43 p.m., 2 cc. dose intravenously. *Result*: Apr. 19, fever, agitation, tremor. Apr. 24, fever down, weakness of legs. May 7, same, killed. *Autopsy*: Mild lesions in medulla, cervical and lumbar cord; none in dorsal cord. Bilateral olfactory neurectomy verified. *Diagnosis*: Mild poliomyelitis.

No. 7-42. *Preparation*: None. *Inoculation*: Apr. 14, 3.46 p.m., 2 cc. dose intravenously. *Result*: Remained well.

No. 7-44. *Preparation*: None. *Inoculation*: Apr. 14, 3.53 p.m., 2 cc. dose intravenously. *Result*: Remained well.

No. 7-21. *Preparation*: Two stage elevation of skin flap in left flank, Mar. 24 and Apr. 7. *Inoculation*: Apr. 14, 4.04 p.m., 2 cc. dose intracutaneously in 10 piqûres in skin flap. *Result*: Apr. 19, fever, paralysis left arm. Apr. 21, prostrate, temperature 99.3°F., killed. *Autopsy*: Extensive lesions in medulla and cord. *Diagnosis*: Severe poliomyelitis.

No. 6-97. *Preparation*: Two stage elevation of skin flap in left flank, Mar. 24 and Apr. 7. *Inoculation*: Apr. 14, 4.07 p.m., 2 cc. dose intracutaneously in 10 piqûres in skin flap. *Result*: Apr. 19, fever, tremor, paralysis of left arm. Apr. 22, prostrate, temperature 96.5°F. Apr. 26, dead. *Autopsy*: Extensive lesions in medulla and cord. *Diagnosis*: Severe poliomyelitis, fatal.

No. 7-45. *Preparation*: Apr. 14, 11 a.m., partial section of left leg at mid-thigh, as in No. 7-22. *Inoculation*: Apr. 14, 4.10 p.m., 2 cc. dose intracutaneously in 10 piqûres in calf of left leg. *Result*: May 1, fever, tremor. May 2, fever, paralysis of both legs. May 4, prostrate, cold. May 6, same, killed. *Autopsy*: Extensive lesions medulla and cord. *Diagnosis*: Severe poliomyelitis.

No. 7-41. *Preparation*: None. Inoculation: Apr. 14, 4.15 p.m., 0.5 cc. dose of 0.05 per cent virus intracerebrally, left. *Result*: Apr. 23, fever, tremor; Apr. 24, weakness of four legs. May 3, better. May 11, discharged. *Diagnosis*: Moderate poliomyelitis.

No. 4-64. *Preparation*: Poliomyelitis, June, 1936, following intracerebral inoculation of Flexner strain (7). *Inoculation*: Apr. 14, 4.17 p.m., 0.5 cc. dose of 0.05 per cent virus intracerebrally, left. *Result*: Remained well for 4 weeks,

later sick, died June 21. *Autopsy:* Old lesions, loss of ganglion cells in cervical and lumbar cord. No recent lesions; generalized caseous tubercles. *Diagnosis:* (a) Immune in test of Apr. 14; (b) tuberculosis.

Experiment 5.—See text.

Experiment 6.—Sept. 13, 1937. Nembutal anesthesia, except in No. 8-10. *Source of virus:* McL. strain; 10 per cent glycerolated human cord.

No. 8-10. *Preparation:* None. *Inoculation:* Sept. 13, 3.14 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. Kept under dial Sept. 13 to 15. *Result:* Sept. 26, clumsy. Sept. 27, fever, paralysis right leg. Sept. 30, both legs weak. Oct. 4, same, lowest temperature 103°F., killed. *Autopsy:* Moderate lesions in medulla, cervical and lumbar cord; none seen in dorsal cord. *Diagnosis:* Mild poliomyelitis.

No. 8-09. *Preparation:* None. *Inoculation:* Sept. 13, 3.23 p.m., 2 cc. dose into left cerebral hemisphere and 5 cc. dose intraperitoneally. *Result:* Sept. 20, fever. Sept. 22, tremor. Sept. 27, right hand weak. Oct. 2, paralysis right hand, legs weak, killed. *Autopsy:* Extensive lesions in cervical cord; moderate lesions in medulla and dorsal cord; lumbar cord not saved. *Diagnosis:* Moderate poliomyelitis.

No. 8-08. *Preparation:* None. *Inoculation:* Sept. 13, 3.26 p.m., 2 cc. dose intracutaneously in 10 piqûres in right flank. Sept. 25, fever, tremor. Sept. 27, legs weak. Oct. 1, better; lowest temperature has been 103°F. Oct. 4, killed. *Autopsy:* Moderate lesions in cervical and lumbar cord; none in dorsal cord; medulla not saved. *Diagnosis:* Mild poliomyelitis.

No. 8-06. *Preparation:* Sept. 13, 2 p.m., skin flap elevated in right flank in one stage. *Inoculation:* Sept. 13, 3.40 p.m., 2 cc. dose intracutaneously in 10 piqûres in skin flap. *Result:* Sept. 14, flap puffy, fluctuant, and oozing. Remained well 4 weeks. *Diagnosis:* No poliomyelitis.

No. 8-07. *Preparation:* Sept. 13, 2.45 p.m., skin flap elevated in right flank in one stage. *Inoculation:* Sept. 13, 3.43 p.m., 2 cc. dose intracutaneously in piqûres in skin flap. *Result:* Sept. 20, fever. Sept. 22, paralysis right leg. Sept. 23, paralysis both legs, weakness of arms and back; temperature 103°F., killed to harvest virus. *Autopsy:* Extensive lesions in cervical and lumbar cord; moderate lesions in medulla and dorsal cord. *Diagnosis:* Moderate poliomyelitis.

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AN UNIDENTIFIED VIRUS PRODUCING ACUTE MENINGITIS AND PNEUMONITIS IN EXPERIMENTAL ANIMALS

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PLATES 2 AND 3

In the winter of 1934, during the course of studies with the virus of epidemic influenza, throat washings from a patient suspected of epidemic influenza were inoculated into ferrets. With repeated passages in ferrets a disease was produced which in the gross resembled influenza virus infection (1, 2). Cross immunity between experimental influenza and the disease in question, however, did not obtain. Furthermore, the pulmonary lesions produced by the agent when introduced intranasally into mice were seen to differ strikingly from those produced by influenza virus. Instead of the edematous bluish red consolidation of influenza in mice (2, 3), the lesions were firm, rubbery, and of a pink pearly grey appearance resembling marble. No difficulty was encountered in maintaining the agent in mouse passage with suspensions of lungs of infected mice, but attempts to perform passive protection tests in mice with serum of ferrets recovered from infection with the homologous agent or from influenza virus infection were unsuccessful. While it was thus impossible to determine with certainty the source of the infectious agent, it seemed most likely to have been derived in the course of passage from a supposedly normal ferret and, consequently, to be of animal origin.

In 1936 an epidemic of respiratory disease clinically resembling epidemic influenza was widespread throughout the United States. An extensive study of material obtained during the outbreak in California (4) was made in these laboratories. Influenza virus was not demonstrated, nor did antibodies to influenza virus develop in the serum of convalescent patients. During the period of the epi-

147

demic, however, an infectious agent identical with that isolated in 1934 was repeatedly recovered from ferrets inoculated with throat washings of the patients. Fresh washings obtained from patients along the Atlantic seaboard as well as washings shipped in 50 per cent glycerine from California and other parts of the country were used. With fresh washings a clinical illness not infrequently occurred in the first ferret inoculated and from the first ferret was without difficulty transferred to mice. Attempts to recover the same agent from normal ferrets were unsuccessful. Attempts to demonstrate the virus in normal mice were also unsuccessful. Moreover, the agent has not been encountered since the spring of 1936 although large numbers of ferrets were inoculated with throat washings the following year in the study of an epidemic of influenza (5, 6).

Regardless of its origin, the frequent occurrence of the infectious agent during the period of study in 1936 made it imperative to determine characteristics which would serve to differentiate the agent from, or to identify it with, known viruses, a class in which it appears to belong. The present report deals with the observations which have been made in these respects.

General Characteristics

The agent to be described belongs, on the basis of information available, in the class of filtrable viruses. Repeated cultures in a wide variety of liquid and solid media, under aerobic and anaerobic conditions, have failed in our hands to reveal a microscopically visible bacterium which would reproduce the experimental disease. Furthermore, careful study of sections of affected organs or impression smears stained by a variety of methods have not ordinarily disclosed recognizable bacteria or parasites. In fixed preparations, however, bodies resembling elementary bodies have been observed.

The agent usually passes a Berkefeld V filter with comparative ease, passes a Seitz filter rarely or in but small amounts, and fails to pass a Berkefeld N filter. The agent filters through graded collodion membranes of 430 mμ average pore diameter but has not penetrated membranes of 250 mμ average pore diameter or less.

With centrifugation in the horizontal centrifuge at 3,000 R.P.M. for 1 hour some slight reduction in titer may occur, but in the high

speed centrifuge (7) at 13,000 R.P.M. almost complete sedimentation occurs in the same length of time.

The agent remains viable in 50 per cent glycerine for 3 to 4 months and rapidly regains full virulence with animal passage.

TABLE I

Pathogenicity of the Virus in Different Animal Species

Animal species	Route of inoculation	Pulmonary consolidation	Paralysis	Fever	Glandular enlargement	Death
Ferret	i.n.	+	0	+	0	+
	s.c. or i.p.	0	0	+	0	0
Mouse	i.n.	+	0		0	+
	i.c.	0	+		0	+
	s.c. or i.p.	0	+		0	+
Rabbit	i.n.					
	i.c.	0	0	+	0	0
	s.c. or i.p.	0	0	+	0	0
	Corneal	0	0	0	0	0
	i. test.	0	0	0	0	0
	i. cut.	0	0	0	0	0
Monkey	i.n.	Negative with throat washings of human patients				
	i.c.	0	+	+	0	0
	s.c. or i.p.	0	0	+	+(s.c.)	0
Guinea pig	i.c.	0	0	+	0	±
	s.c.	0	0	+	+	±
	i.p.	0	0	+	0	±

i.c. = intracerebral.   i. cut. = intracutaneous.
i.p. = intraperitoneal.   i. test. = intratesticular.
i.n. = intranasal.   s.c.   = subcutaneous.

Attempts to maintain the agent in chick embryo-Tyrode's tissue culture medium have not been fruitful, but it is readily maintained by passage on the chorio-allantoic membrane of the developing egg.

The virus is pathogenic for ferrets, mice, monkeys, and within certain limits for guinea pigs and rabbits. These effects are summarized in Table I.

Infection of Animals

Experimental Disease in Ferrets.—Ferrets of all ages have been found to be susceptible. Passage of the virus in ferrets has ordinarily been carried out by the intranasal route.

A 10 per cent suspension of the lung and turbinate tissue of an infected ferret is inoculated intranasally into a normal ferret anesthetized with ether. After an incubation period of 24 to 48 hours, a sharp rise in temperature to 105–106°F. or higher may be observed. The temperature may be sustained for 4 to 5 days and is accompanied by weakness, loss of appetite, irritability, nasal discharge, and labored breathing. The majority of animals have been sacrificed on the 4th or 5th day and not infrequently the animal is moribund at this time. In others, fever may persist for 7 to 10 days with continuation of respiratory distress during the entire period but with ultimate recovery. At other times little fever or few signs of infection are observed, but autopsy reveals pulmonary involvement. Thus it is seen that considerable variation in the disease picture occurs. No evidence of involvement of the central nervous system has been observed following intranasal infection. Diarrhea is not uncommon and considerable dehydration occurs.

The most prominent pathological feature of the disease following intranasal infection of ferrets is an extensive edematous pulmonary involvement resembling in many respects that produced by influenza virus (2, 8). In general, however, the parenchyma of the lung is firmer than in the case of experimental influenza. Cultures of the lung are usually bacteriologically sterile. There is, in addition, congestion of the nasal tissues and a moderate amount of mucoid exudate. No marked abnormality of the remaining organs has been observed.

Following intraperitoneal inoculation of virus the ferret may exhibit marked weakness, sustained fever, and diarrhea for 3 to 10 days, but recovery has always resulted.

Large doses of infectious material given subcutaneously to the ferret have failed to produce objective evidence of infection other than a febrile response of 2 to 3 days' duration.

Experimental Disease in Mice.—*Intranasal inoculation*: The experimental infection is readily transferred to mice of all ages by the intranasal inoculation of suspensions of infected ferret lung. It requires little adaptation and can be transmitted serially in mice with little or no difficulty.

With a 10 per cent suspension of infected mouse lung as inoculum death of the inoculated mice occurs in 2 to 4 days; but when the dosage is decreased to 0.01

per cent or less, death is delayed for as long as 15 to 21 days and only rarely is there full recovery. Infected mice exhibit ruffled fur, respiratory distress, sticky eyes, and audible, wet respirations. No neurological signs occur.

The typical lesion occurs in the lungs which present a firm, rubbery, pinkish grey pneumonia involving most of the lung. At times the lung has an almost cartilaginous consistency when cut, and considerable edema fluid exudes. After intranasal inoculation the virus is recovered not only from the lung but from the blood, spleen, and liver as well.

Intracerebral inoculation: When the virus is introduced into the brains of mice an entirely different disease picture is produced. In a dilution of 1:1,000,000, virus injected intracerebrally causes paralysis and death of the animal in 6 to 10 days. Following infection with 10 per cent or 1 per cent suspensions of virus material, death ordinarily occurs in 2 to 3 days.

The first evidence of infection after intracerebral inoculation is a decrease in the activity of the mouse and the assumption of a crouching attitude. The animal scratches its nose constantly. The eyelids become tightly glued. The tail becomes hypersensitive, and shortly there is noted a flaccid paralysis of the hind limbs which drag along to one side while the animal walks with its fore legs. The hair over the posterior half of the body lies flat, in contrast to the erect state of the hair over the anterior portion of the body. Soon the animal becomes prostrate, struggling feebly and unable to right itself if placed in the supine position although the fore limbs are usually not paralyzed. Terminal convulsions may occur. The rate of development of symptoms varies with the infecting dose and the more typical progress of the disease is observed in animals which receive the smaller doses. An occasional mouse recovers after paralysis of indefinite duration. In contrast to the results of intranasal inoculation no pulmonary lesions are seen following intracerebral infection. The brain, however, is swollen and edematous and the characteristic lesion is a generalized meningitis.

Intraperitoneal and subcutaneous inoculation: The intraperitoneal inoculation of mice results in a somewhat varied disease. The animal becomes sick, and the abdomen is swollen and contains a fair amount of cloudy fluid and considerable fibrin. With large intraperitoneal doses of virus 50 per cent or more of the mice die with paralysis similar to that which occurs following intracerebral inoculation. If broth or starch is injected into the brain at the time virus is given intraperitoneally, only the exceptional mouse survives. Pulmonary lesions are not usually seen.

The subcutaneous inoculation of mice with strong virus suspensions

usually results in the depilation of the skin over the site of injection and the development of a firm granulomatous subcutaneous swelling. The skin may become contracted and break down. Approximately 10 to 15 per cent of mice so inoculated develop typical paralysis of the posterior quarters and die. A certain number recover from the paralysis while the majority reveal no clear evidence of central nervous system involvement. They do not develop pneumonia.

Experimental Disease in Monkeys.—Two monkeys (one *M. rhesus* and one *M. cynomolgos*) were inoculated intracerebrally with mouse passage virus. The course of the disease was quite similar in both instances. After an interval of 48 hours a brisk rise of temperature to 104°F. occurred. At the same time a flaccid paresis of one arm was noted. Fever and paralysis persisted for little more than 48 hours after which the animals rapidly recovered.

Cerebrospinal fluids obtained at the height of the disease by cisternal puncture contained, respectively, 500 and 1,000 cells, practically all of which were lymphocytic in type. Virus was recovered from the spinal fluid upon the intracerebral inoculation of the fluid into mice.

One of the animals which recovered after intracerebral inoculation was tested for immunity to infection with poliomyelitis virus and succumbed.

The virus was given to one monkey (*M. cynomolgos*) subcutaneously in the groin. On the 2nd day following inoculation there was slight enlargement of the inguinal gland, loss of appetite, and listlessness. On the 3rd day the temperature reached 105°F., then within 12 hours rapidly returned to normal limits. The animal recovered completely without evidence of involvement of the central nervous system.

Experimental Disease in Guinea Pigs and Rabbits.—The intracerebral inoculation of guinea pigs with the virus usually elicits fever and loss of weight as the only objective evidence of infection. Following a quiescent period of 48 to 72 hours a sharp rise of temperature to 105° or 106°F. is observed. The fever persists for 4 to 5 days after which relatively prompt recovery usually ensues. A small percentage of the animals die 5 to 6 days after inoculation, without paralysis.

When the virus is given subcutaneously to guinea pigs, thickening of the skin and induration of the regional lymph nodes occur. These

observations are somewhat obscured, however, by the frequent appearance of intercurrent infection in the stock of guinea pigs employed. Plantar inoculations were uniformly negative.

In rabbits the intracerebral inoculation of virus also causes a peak of fever, usually without paralysis or other evidence of disease. Following intraperitoneal inoculation a brief febrile reaction may occur but no other characteristic signs of infection are observed. Subcutaneous injection may cause fever and local cutaneous infiltration. Inoculation of virus on the scarified cornea, into the testicles, and into the skin has elicited no significant changes.

Pathology

In the ferret significant pathological changes have been primarily limited to the lungs and have been studied only after intranasal infection.

As previously mentioned, a pneumonia of variable extent occurs. The involved lobes are plum-colored, firm, and distended with edema fluid which flows freely from the cut surface of the bronchi. At times a clear albuminous fluid is present in the pleural cavity.

Microscopic examination reveals a pneumonia similar to that produced by the viruses of influenza (2, 8), psittacosis (9), or Rift Valley fever (10). There is edema of the bronchial walls but little or no desquamation of the bronchial epithelium; in fact, it appears somewhat hyperplastic. Exudate may be formed in the lumen of the bronchus. The vascular endothelium appears swollen and unusually prominent but hyperemia is not an outstanding feature. The alveolar walls are edematous and densely infiltrated with large mononuclear cells containing large pale nuclei (Figs. 1 and 2). These cells seem almost to form a lining of the alveolar spaces and the appearance of the lung in places approaches the adenomatous.

The alveolar spaces are distended and contain a moderate cellular exudate consisting primarily of large pale-staining mononuclear cells. Polymorphonuclear leukocytes are not prominent. Fibrin is usually not observed, and edema fluid, despite the large amount which seeps from the cut lung, is relatively scanty. In general, however, the lung presents a picture of a more proliferative type than that observed in infection with the virus of influenza.

The pathology in mice varies with the route of inoculation of the virus.

The pulmonary lesions are the outstanding feature after intranasal inoculation (Fig. 3). The early lesions are characterized by a certain degree of hyperemia

but especially by a rich effusion of edema fluid which fills the alveolar spaces over a comparatively large area. While edema of the alveolar walls is of moderate extent a few large mononuclear cells are observed. A rich cellular exudate resembling true consolidation later appears diffusely throughout the lung. The cells are predominantly large mononuclear in type but lymphocytes and polymorphonuclear leukocytes are scattered throughout. Fibrin deposits may occur. Hyperemia and extravasation of erythrocytes may appear. At times the exudate is so extensive as to make identification of the alveolar walls difficult. The latter, however, are swollen and infiltrated with large cells. Still later the lung shows dense infiltration of the parenchyma throughout and the alveoli are densely packed with cellular exudate. The appearance of the mouse lung is in general quite different from that observed in infection with influenza virus.

In mice which develop paralysis following intracerebral or intraperitoneal inoculation, an extensive meningitis and choroiditis is observed over the brain and spinal cord (Fig. 4). No perivascular cuffing or other evidence of infection is seen in the parenchyma of the brain or spinal cord. Because of the type of paralysis it was thought that damage to anterior horn cells of the spinal cord should be detectable, and in some sections alterations suggesting cytolysis in these cells were observed. Later studies, however, and examination of normal spinal cords did not substantiate these impressions (Fig. 5). The meningeal and choroidal reaction is of a mixed mononuclear and polynuclear type, the proportion of the latter cells being apparently related somewhat to the acuteness of the disease. The meningitis is diffuse over the dorsal and basilar aspects of the central nervous system. The ganglion cells do not appear to be damaged. Inclusion bodies have not been detected.

In the peritoneal exudate of mice which are inoculated intraperitoneally a rich collection of large mononuclear cells is found. Search for inclusion bodies in these cells has been unsuccessful.

The brains of monkeys infected with the virus were not examined.

In rabbits and guinea pigs a subcutaneous granulomatous lesion may develop after subcutaneous injection. There may also be hemorrhagic extravasation into the indurated tissue. Regional lymph nodes become swollen, hyperplastic, and firm.

Immunity

Ferrets which recover from intranasal infection are usually resistant to reinoculation.

Mice which recover after intranasal infection are resistant to reinoculation by the same route. Mice which recover after intraperitoneal or subcutaneous inoculation of virus are firmly immune

to subsequent intracerebral inoculation, regardless of whether or not they exhibited any evidence of infection as a result of the primary inoculation, but these mice are fully susceptible to the virus administered intranasally. However, efforts to recover virus from the brain or spinal cord of immune animals have proved unsuccessful. The immunity developed to one strain of virus is fully effective against all other strains of the same virus.

Attempts to study serological immunity by means of passive protection tests in mice have been somewhat difficult. Serum from recovered animals or from rabbits immunized by intraperitoneal inoculation fails to protect test animals when serum-virus mixtures are injected intracerebrally or intranasally. When serum-virus mixtures containing 10 per cent virus are given intraperitoneally the mortality is high, while with weaker concentrations of virus the percentage of survivors is high. When broth is given intracerebrally to mice before the administration of virus intraperitoneally, a uniformly high mortality occurs with the weaker dilutions of virus. Consequently the following procedure was adopted. A 3 per cent suspension by weight of infected mouse brain was made in 10 per cent horse serum-saline. To 1 volume of the virus suspension were added 2 volumes of the serum to be tested, and 0.3 cc. of the mixture (final concentration of virus equals 1 per cent) was injected intraperitoneally into mice which had 1 hour previously been given intracerebrally 0.03 cc. of sterile broth. The mice were observed for 10 days and survival or death of the animals was recorded. In Table II are presented results typifying those which have been obtained.

It has been possible to show by this means that the serum of known immune animals contains antibodies which protect the test mice from fatal infection. The margin of safety is narrow, however, since it is not always possible to obtain duplicate results with the same serum in different tests. Nevertheless, the sera of recovered ferrets and rabbits immunized by intraperitoneal inoculation have been shown to possess protective antibodies, whereas normal ferret and rabbit sera have usually been devoid of any protective capacity. In no instance, however, has serum from a human individual been found to exert more than a suggestive effect. This is true of convalescent sera from the patients studied during the respiratory epidemic in

the early months of 1936 (4). Since it has so far been possible clearly to demonstrate immune substances only in the serum of animals recovering from rather severe infections or repeated inoculations, the difficulty in evaluating minor effects is obvious. As a result, the procedure has failed to yield significant information as to the origin of the infectious agent.

TABLE II

Protection Test in Mice with Sera from Various Sources

Serum		Mouse No.								Protection
Source	Nature of immunity	1	2	3	4	5	6	7	8	
Ferret 4-14	Influenza	d5	d5	d6	d6	d6	d9	d10	d10	None
Ferret 5-96	M-P virus	d5	d5	d5	d6	d9	S	S	S	Suggestive
Ferret 3-30	M-P virus	S	S	S	S	S	S	S	S	Complete
Ferret 4-06	M-P virus	S	S	S	S	S	S	S	S	Complete
Rabbit	Normal	d7	d7	d7	d7	d8	d8	d9	d9	None
Rabbit 1-35	M-P virus	d6	S	S	S	S	S	S	S	Good
Ferret 4-01	Influenza	d5	d5	d6	d6	d8	d8	d9	S	None
Ferret 6-11	M-P virus	d9	S	S	S	S	S	S	S	Good
Ferret 5-99	Influenza	d5	d7	d7	d8	d8	d9	d9	S	None
Ferret 2-64	M-P virus	S	S	S	S	S	S	S	S	Complete
Human	S.S.* acute	d5	d5	d6	d6	d6	d7	d8	d9	None
Human	S.S.* convalescent	d7	d7	d7	d8	d8	S	S	S	Suggestive
Culture broth		d6	d6	d7	d7	d8	d8	d10	S	None

d5 = mouse died 5th day; S = survived.

M-P = the virus described in this report, meningo-pneumonitis.

* Human serum from patient whose throat washings were given to ferret from which original M-P virus was obtained.

Differential Diagnosis of the Virus

It has not been possible to identify the virus reported in this paper with viruses already known. It most closely resembles 4 viruses which have been described: (*a*) encephalomyelitis of mice, described by Theiler (11); (*b*) psittacosis (9, 12, 13); (*c*) lymphocytic chorio-meningitis (14, 15, 16); and (*d*) lymphogranuloma inguinale (17).

From encephalomyelitis of mice the virus is distinguished by its much larger size and by differences in the pathological findings in mice, as well as by its pathogenicity for monkeys.

The agent is differentiated from that of psittacosis by the absence of the typical hepatic necrosis produced in mice and guinea pigs infected with psittacosis. Furthermore, the characteristic picture of the nervous disease in mice following intraperitoneal or subcutaneous inoculation of the virus has never been reported in psittacosis. Nevertheless, certain of the features of psittacosis in rabbits, guinea pigs, and monkeys bear suggestive resemblances to the picture herein reported, while the size of the psittacosis agent is in the same general range as that observed in the present instance.

The virus has many resemblances to the virus of lymphocytic choriomeningitis. The disease produced by the new virus after intracerebral inoculation in mice, although much more rapidly fatal, produces a choriomeningitis very similar to that of lymphocytic choriomeningitis. The pulmonary lesions produced in mice by the former have, however, no counterpart in reported studies with the virus of lymphocytic choriomeningitis. The latter produces a more uniformly fatal infection in guinea pigs. Both viruses are capable of producing a meningeal infection in mice. It has been possible[1] on two occasions to test cross immunity in mice with the two viruses. Mice known to be immune to lymphocytic choriomeningitis have not been immune to the unknown virus, nor have mice immune to the latter virus been resistant to lymphocytic choriomeningitis. Thus, while resemblances exist between the two viruses, the evidence indicates that they are different.

Many characteristic effects of the virus in question resemble those produced by the virus of lymphogranuloma inguinale (17). The pathology in the central nervous system of mice is similar with the two viruses. Paralysis and death of mice after subcutaneous inoculation have not been reported with lymphogranuloma inguinale nor have the typical pulmonary lesions which occur in mice and ferrets after intranasal inoculation of the new virus been observed with lymphogranuloma inguinale virus. The virulence of strains of lymphogranuloma inguinale virus obtained in America is much lower and the immunity produced less striking. Nauck and Malamos (18) have reported the recovery of strains of virus of lymphogranuloma inguinale which more nearly approach the virulence of the virus here

[1] Through the courtesy of Dr. T. F. McN. Scott and Dr. T. M. Rivers.

described. Even these strains, however, survive only a few days in 50 per cent glycerine and produce neither nervous symptoms nor immunity with subcutaneous or intraperitoneal inoculation of mice. They do produce a typical disease in *M. rhesus*. The size of the two viruses is similar. Moreover, granulomatous infiltrations in the skin of mice, guinea pigs, and rabbits are produced by both. The granules observed in lymphogranuloma infection (19) have not, however, been detected in the present studies.

It has been possible to test cross immunity in mice immune to the respective viruses. None was detectable. Mice immune to lymphogranuloma inguinale were fully susceptible to the new virus and vice versa. Furthermore, the type of paralysis produced by the unknown virus was recognized to be decidedly different from that seen in the disease produced by lymphogranuloma inguinale virus. It was interesting to observe that, when tested with lymphogranuloma inguinale, mice immune to the unknown virus died with a paralysis exactly similar to that produced by the new virus in susceptible animals. This suggests that the sites damaged by the virus at the time of immunization were again attacked by the heterologous test virus to produce a clinical disease picture more closely resembling the original than the test infection.

Further cross immunity tests were conducted by Dr. Marion Howard of Yale University Medical School, who found that her mice immunized to strains of lymphogranuloma inguinale were completely susceptible to the unknown virus. In addition, Dr. Howard prepared antigen from infected mouse brains for Frei tests. The material failed to elicit the Frei reaction in patients known to be positive reactors, while tests with lymphogranuloma inguinale virus preparations were typically positive. This evidence seems almost conclusively to eliminate lymphogranuloma inguinale from further consideration unless antigenically distinct strains exist. It is quite possible, of course, that despite the negative results of cross immunity tests, the virus herein described is related either to lymphocytic choriomeningitis or to lymphogranuloma inguinale. If so, it possesses certain striking pathogenic differences from the known strains of the two viruses as well as sharply different im-

through the kindness of Dr. A. W. Grace and Mrs. F. H. Suskind of The New York Hospital.

munological characters. In any event, it must be noted that the virus was not obtained from the usual source of lymphocytic choriomeningitis virus, *i.e.*, the spinal fluid; nor of lymphogranuloma inguinale, *i.e.*, buboes. Its exact source is at present uncertain, but it is possible to assert that the new virus was derived either from the throat washings of patients suffering from an epidemic disease clinically resembling epidemic influenza or from ferrets inoculated with this material. In the present state of the studies the virus described in this report appears to possess more characteristics in common with lymphogranuloma inguinale than with lymphocytic choriomeningitis. Because of the two most prominent pathological effects which it produces in experimental animals, it is suggested that for purposes of identification the agent be called the *virus of acute meningo-pneumonitis*.

SUMMARY

An infectious agent is described which belongs apparently to the class of filtrable viruses, but which, on the basis of the evidence at hand, is not to be identified with any virus previously described.

The virus has multiple tropisms and is pathogenic for mice, ferrets, and monkeys of both *M. rhesus* and *M. cynomolgos* species. Intranasal infection of mice and ferrets causes extensive pneumonic lesions of fatal severity. Intracerebral inoculation of the virus produces in monkeys a lymphocytic choriomeningitis from which the animal recovers, while in mice a rapidly fatal choriomeningitis is produced. Fatal paralysis occurs in a moderate proportion of mice which receive the virus by intraperitoneal or subcutaneous routes, while the remainder become immune to the intracerebral test but not to the intranasal test. Subcutaneous inoculation of mice, monkeys, ferrets, rabbits, and guinea pigs causes local granulomatous induration of the skin with enlargement of the regional lymph nodes.

The virus was repeatedly recovered in 1936 from ferrets inoculated with throat washings of patients suffering from an epidemic disease clinically indistinguishable from epidemic influenza. It is impossible, however, to conclude whether the virus is of ferret or human origin.

Although possessing many features in common with the virus of lymphocytic choriomeningitis and the virus of lymphogranuloma inguinale, cross immunity tests have failed to yield any evidence that

the new agent is immunologically related to either of the aforementioned viruses.

For purposes of identification the name *virus of acute meningo-pneumonitis* is suggested.

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EXPLANATION OF PLATES

PLATE 2

FIG. 1. Cross section of lung of ferret 4 days after intranasal infection with virus. There is almost complete consolidation of the lobe, especially dense in the rounded aspect. Giemsa. × 8.

FIG. 2. High power magnification of lung shown in Fig. 1. There is thickening and infiltration of the alveolar walls which are somewhat obscured by the cellular exudate. The pneumonic exudate is composed primarily of large and small mononuclear cells interspersed with polymorphonuclear leukocytes. Giemsa. × 450.

FIG. 3. Lung of mouse sacrificed 4 days after intranasal infection with virus. The marked induration and thickening of the alveolar walls is apparent. The rich cellular exudate is characterized by large pale-staining mononuclear cells although other types of cells are numerous. Giemsa. × 450.
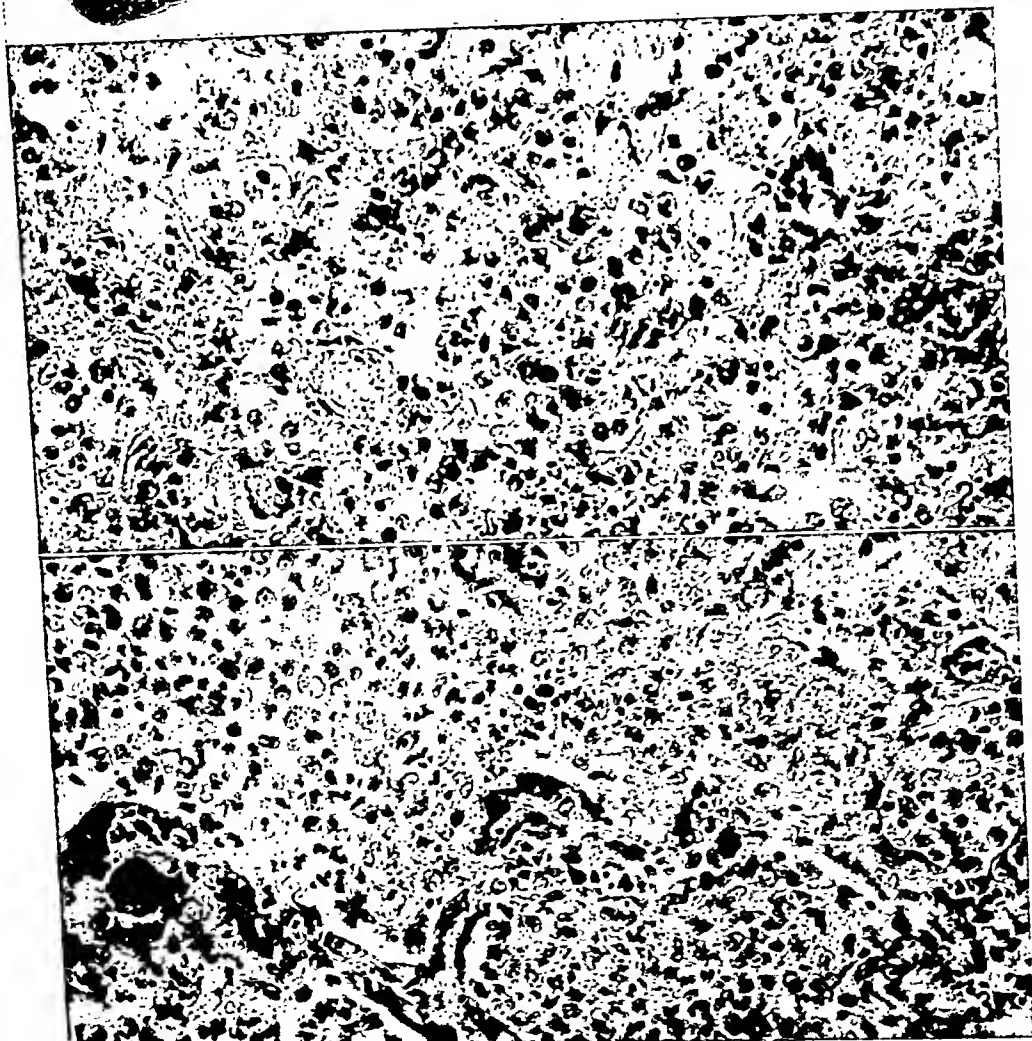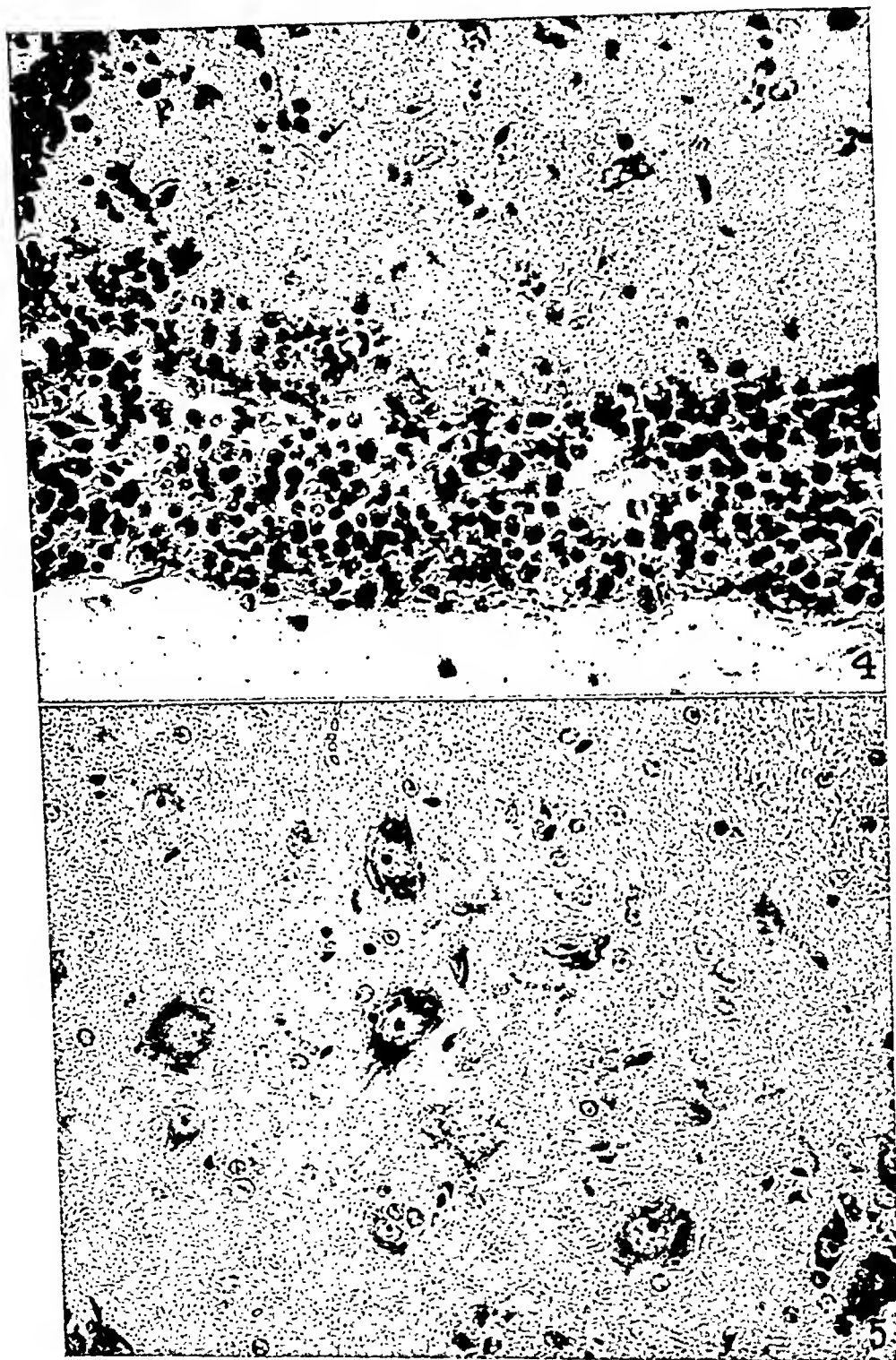
(Francis and Magill: Virus of meningitis and pneumonitis)

PLATE 3

FIG. 4. Section of brain of mouse sacrificed 3 days after intracerebral inocula-
tion of virus. There is a marked leptomeningitis predominantly mononuclear in
type but a sprinkling of polymorphonuclear leukocytes is also present. Note
the absence of parenchymal lesions. Giemsa. × 450.

FIG. 5. Anterior horn of lumbar portion of spinal cord of mouse which died with
paralysis of hind quarters 3 days after intracerebral inoculation of virus. The
pyramidal cells are apparently undamaged nor is there any evidence of paren-
chymal injury to the cord. Giemsa. × 450.

(Francis and Magill: Virus of meningitis and pneumonitis)

THE EFFECT OF CORTICOSTERONE AND RELATED COMPOUNDS ON THE RENAL EXCRETION OF ELECTROLYTES*

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In earlier studies (1–3) we have observed that subcutaneous injections of suprarenal cortical extract affected the renal excretion of sodium, chloride and potassium in normal subjects and in normal dogs as well as in suprarenalectomized dogs and patients suffering from Addison's disease. With the isolation of steroid compounds from the suprarenal cortex (Kendall, and his coworkers, Reichstein, Wintersteiner and Pfiffner, and Grollman (4)) it has become possible to test crystalline preparations for their effect on the renal excretion of electrolytes and to determine whether this effect parallels their potency in maintaining suprarenalectomized animals. In addition to crystalline compounds[1] derived from the suprarenal cortex we have tested a synthetic compound (desoxy-corticosterone acetate) prepared by Steiger and Reichstein (5).

Methods

Male dogs (approximately 10 kilos) were maintained in metabolism cages under constant dietary conditions. The routine care of the dogs and the conduct of the metabolic studies have been described elsewhere (3). Bilaterally suprarenalectomized dogs were used for the studies on suprarenal insufficient animals.[2]

These animals were maintained on exactly the same regimen as the normal dogs, with the single exception that suprarenal cortical extract[3] in maintenance doses was injected twice daily during the intervals between experiments. All of the animals were catheterized at the completion of each 24 hour period and the 24 hour urine specimens were collected and preserved with toluene. Specimens of blood were withdrawn under oil from the jugular veins, care being taken to avoid stasis. Urine and blood specimens were analyzed for sodium (6), chloride (7), potassium (8, 9), inorganic phosphate (10), total urine nitrogen and serum protein nitrogen (macro Kjeldahl), non-protein nitrogen (11), blood sugar (Folin-Wu) and CO_2 combining power of the serum (12).

One-half of the total daily quantity of extract or crystalline compound was injected at 10 a.m. and the remainder at 5 p.m. The possible effect of the solvents employed was controlled by experiments in which an equal quantity of either the saline solution or mazola oil was injected.

OBSERVATIONS

Suprarenal Cortical Extracts.—The injection of suprarenal cortical extracts produced a marked retention of sodium and chloride and an increased renal excretion of potassium in normal dogs (Table I). In this experiment 10 cc. of extract contained 10 mg. of solid material. An aliquot of this same preparation was extracted with ethyl acetate according to the method described by Grollman (13).[4] This procedure yielded an inactive[5] fraction containing 8 mg. of the original solid material and an active[5] fraction containing 2 mg. of the original solids. The active fraction produced a marked decrease in the renal excretion of sodium and chloride when injected into a normal dog (Table II), whereas the inactive fraction failed to produce this effect (Table III). The injection of either the active or the inactive fraction produced a potassium diuresis (Tables II and III). It therefore appeared desirable to test possible contaminants of the extract for a potassium diuretic effect. Experiments in which freshly prepared dilute solutions of epinephrin (1:250,000) were injected demonstrate the effectiveness of this substance in producing both a potassium and

[3] We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, Illinois, for the generous supply of suprarenal cortical extract which was provided for this study.

[4] We are indebted to Dr. Arthur Grollman for carrying out this procedure and also for the gift of dehydro-corticosterone.

[5] The use of the term active or inactive in this study, refers specifically to the effectiveness of the preparation in maintaining suprarenalectomized dogs.

TABLE I

The Effect of the Subcutaneous Injections of Suprarenal Cortical Extract (Aqueous Solution) on the Renal Excretion of Electrolytes in the Normal Dog (Dog 1)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m. eq.*	*m. eq.*	*m. eq.*	
Control............	455	51.6	49.8	16.5	
Treated............	545	30.5	34.0	21.3	10 cc. of suprarenal cortical extract*
Control............	625	86.6	79.2	10.3	

* 10 cc. of suprarenal cortical extract which contained a total of 10 mg. of solid material.

TABLE II

The Effect of the Subcutaneous Injections of the Active Fraction of Suprarenal Cortical Extract (Aqueous Solution) on the Renal Excretion of Electrolytes in the Normal Dog (Dog 2)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m. eq.*	*m. eq.*	*m. eq.*	
Control............	735	62.7	58.2	21.4	
Treated............	490	49.2	50.8	26.1	Active fraction of original extract*
Control............	640	64.2	62.0	17.6	

* This fraction represents a total of 2 mg. or 20 per cent of the solid material which the original extract contained.

TABLE III

The Effect of the Subcutaneous Injections of the Inactive Fraction of Suprarenal Cortical Extract (Aqueous Solution) on the Renal Excretion of Electrolytes in the Normal Dog (Dog 3)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m. eq.*	*m. eq.*	*m. eq.*	
Control............	540	51.1	56.0	15.8	
Treated............	585	54.4	58.8	18.7	Inactive fraction of original extract*
Control............	585	55.0	59.0	13.9	

* This fraction represents a total of 8 mg. or 80 per cent of the solid material which the original extract contained.

a sodium diuresis (Table IV). It appears that in suprarenal cortical extracts the sodium and chloride retaining effect follows the cortical hormone activity, whereas the potassium diuresis may be due to the effect of at least two substances, *i.e.* suprarenal cortical hormone and traces of epinephrin.

Crystalline Compounds Derived from the Suprarenal Cortex.—It has been reported by Reichstein *et al.* (14) and Kendall *et al.* (15) that the

TABLE IV

The Effect of the Subcutaneous Injections of a Dilute Solution of Epinephrin (Aqueous Solution) on the Renal Excretion of Electrolytes in the Normal Dog (Dog 4)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m. eq.*	*m. eq.*	*m. eq.*	
Control............	570	54.2	55.6	7.5	
Treated............	555	66.3	57.8	15.6	Epinephrin*
Control............	530	43.9	46.9	8.2	

* 12.5 cc. of a 1:250,000 solution of epinephrin, freshly prepared, were injected twice daily.

TABLE V

The Effect of the Subcutaneous Injections of Corticosterone in Oil on the Renal Excretion of Electrolytes in the Normal Dog (Dog 1)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m. eq.*	*m. eq.*	*m. eq.*	
Control............	450	54.3	55.2	19.0	
Treated............	515	45.8	48.9	22.7	4 mg. corticosterone*
Control............	635	64.9	63.3	16.0	

* In 3 cc. of mazola oil.

crystalline compounds, corticosterone (Δ⁴-pregnene-11, 21-diol-3, 20-dione) and dehydro-corticosterone (Δ⁴-pregnene-21-ol-3, 11, 20-trione) maintain suprarenalectomized animals in good condition. The injection of corticosterone (4 mg.) into a normal male dog resulted in a marked decrease in the renal excretion of sodium and chloride and an increased potassium excretion (Table V). Dehydro-corticosterone was found to produce a similar effect in approximately the same dosage (Chart 1). The effect of these two compounds on the renal
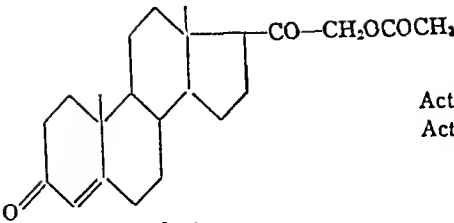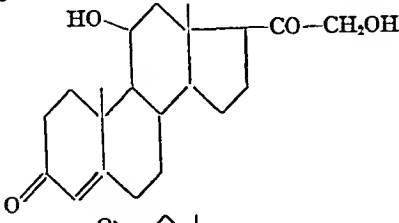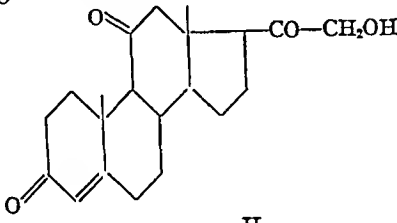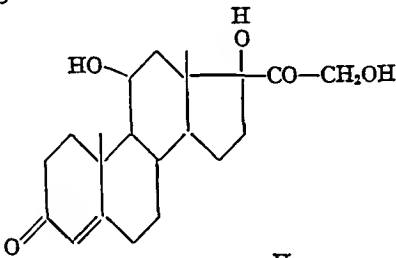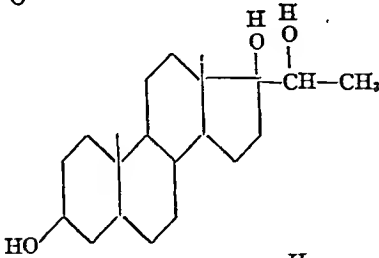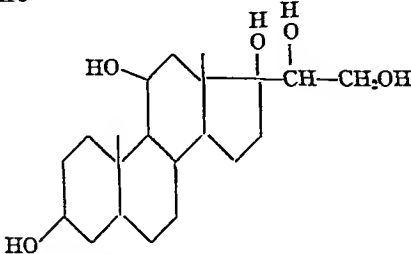
Compound	Structure	Activity	Sodium Retaining Effect in Normal Dog
Desoxy-cortico-sterone acetate	CO—CH₂OCOCH₃	Active (A) Active (B) 0.3 mg.	Present (0.5–1 mg.)
Corticosterone	HO CO—CH₂OH	Active (A) Active (B) 0.8 mg.	Present (4 mg.)
Dehydro-corticosterone	CO—CH₂OH	Active (A) Active (B) > 0.8 mg.	Present (4 mg.)
Compound M	HO CO—CH₂OH	Active (B) 1.5 mg.	Absent (8 mg.)
Compound J	CH—CH₃	Inactive (B) 2.0 mg.	Absent (10 mg.)
Compound A	HO CH—CH₂OH	Inactive (A) Inactive (B) 2.0 mg.	Absent (10 mg.)

CHART 1. The correlation between suprarenal cortical hormone activity and sodium a chloride retaining effect.

A, maintenance of suprarenalectomized dog.   B, Everse-de Fremery te

165

excretion of sodium, chloride and potassium was similar to that noted when suprarenal cortical extract was injected.

Three other crystalline compounds derived from the suprarenal cortex have been tested on normal dogs (Chart 1). Allopregnane-3, 11, 17, 20, 21-pentol (compound A, Reichstein and Wintersteiner, compound D, Kendall), which has been shown previously to be inactive in both the Everse-de Fremery test in doses of 2 mg. per day (16, 17) and the suprarenalectomized dog (18), was found to have no sodium and chloride retaining effect when injected in doses up to 10 mg. Allopregnane-3, 17, 20-triol (compound J, Reichstein) (19), which is also inactive in the Everse-de Fremery test in doses of 2 mg. daily (17), had no sodium and chloride retaining effect when injected in doses up to 10 mg. The injection of Δ^4-pregnene-11, 17, 21-triol-3, 20-dione (compound M, Reichstein) did not produce a sodium and chloride retention in doses up to 8 mg. although it appears to be active in the Everse-de Fremery test in doses of 1.5 mg. daily (17). No reports are available as to the potency of this compound in maintaining suprarenalectomized dogs. In the normal dog the injection of this compound consistently increased the renal excretion of potassium in the higher doses (5 and 8 mg.).

A Synthetic Compound, Desoxy-Corticosterone Acetate.—Desoxy-corticosterone acetate (Δ^4-pregnene-21-ol-3, 20-dione acetate, 21-acetoxy-progesterone) has been prepared by Steiger and Reichstein (5) from stigmasterol. This substance has been shown by Reichstein to be capable of maintaining suprarenalectomized dogs, and in a dosage of 0.3 mg. daily gives a positive Everse-de Fremery test (17). A single injection of 1 mg. of desoxy-corticosterone acetate produced a very marked retention of sodium and chloride and an increased renal excretion of potassium in a normal dog (Table VI). In this respect desoxy-corticosterone acetate appeared to be much more active than corticosterone or dehyro-corticosterone (Chart 1).

Further studies with desoxy-corticosterone acetate were carried out on a suprarenalectomized dog (Chart 2).

When a quantity of suprarenal cortical extract equivalent to 600 gm. of fresh cortex per day was injected, the 24 hour renal excretion of sodium and chloride amounted to 40 and 45 m.eq. respectively (intake 62 m.eq. of sodium and 63 m.eq. of chloride) and the animal gained weight rapidly. Desoxy-corticosterone acetate

(1 mg. daily) was then substituted for the suprarenal cortical extract. The injection of this quantity of synthetic substance was associated with a renal excretion of 48 m.eq. of sodium and 52 m.eq. of chloride, the animal apparently being in positive sodium and chloride balance and the weight increase continuing. During the 7 days of treatment with desoxy-corticosterone acetate, the animal ate well, appeared well, gained weight (0.35 kilo), maintained normal blood levels of sugar, non-protein nitrogen, serum sodium and chloride, CO_2 combining power and plasma volume (hematocrit and serum protein). On the 8th day the treatment was discontinued; diuresis was noted during the first 24 hours and the renal excretion of sodium and chloride rose from an average daily level of 48 and 52 m.eq. respectively to 70 and 85 m.eq. respectively. The excretion of potassium and phosphate was decreased. The animal lost weight (0.25 kilo) and on the morning of the 3rd day appeared to be weak and refused food. At this time it was

TABLE VI

The Effect of the Subcutaneous Injection of Desoxy-Corticosterone Acetate in Oil (1 Mg. Given in a Single Injection) on the Renal Excretion of Electrolytes in the Normal Dog (Dog 1)

24 hr. period	Urine volume	Sodium	Chloride	Potassium	Treatment
	cc.	*m.eq.*	*m.eq.*	*m.eq.*	
Control.............	500	60.0	58.8	16.8	
Treated.............	525	41.4	49.1	21.4	1 mg. of desoxy-corticosterone acetate*
Control.............	715	71.6	66.8	16.9	

* In 2 cc. of mazola oil.

found that the non-protein nitrogen of the blood had risen from its original level of 30 mg. per cent to 60 mg. per cent, the cell volume (hematocrit) had increased from 30 per cent to 40 per cent and the serum concentration of sodium, chloride and bicarbonate were decreased, the serum sodium falling from a level of 150 m.eq. to 130 m.eq. per liter of serum (20, 21). The fasting blood sugar level was maintained during this period. Since a crisis appeared to be impending, the animal was given 1 mg. of desoxy-corticosterone acetate daily for 3 days. During this period a marked decrease in urine volume was observed, associated with a retention of sodium and chloride. The excretion of potassium and phosphate was increased. No further weight loss was observed and the animal's appetite and strength improved markedly. The non-protein nitrogen of the blood, the plasma volume (hematocrit and serum protein) and the serum concentration of sodium and chloride returned to normal. Further experiments of this nature were impossible at this time because of the very limited supply of desoxy-corticosterone acetate. The animal was then maintained on suprarenal cortical extract. After a period of

10 days on extract during which the animal appeared to be in excellent condition, extract was discontinued and the animal died 3 days later, thus establishing the fact that suprarenalectomy had been complete (as shown by autopsy) and that it was not possible to maintain the animal on the routine metabolic regimen without the addition of cortical extract.
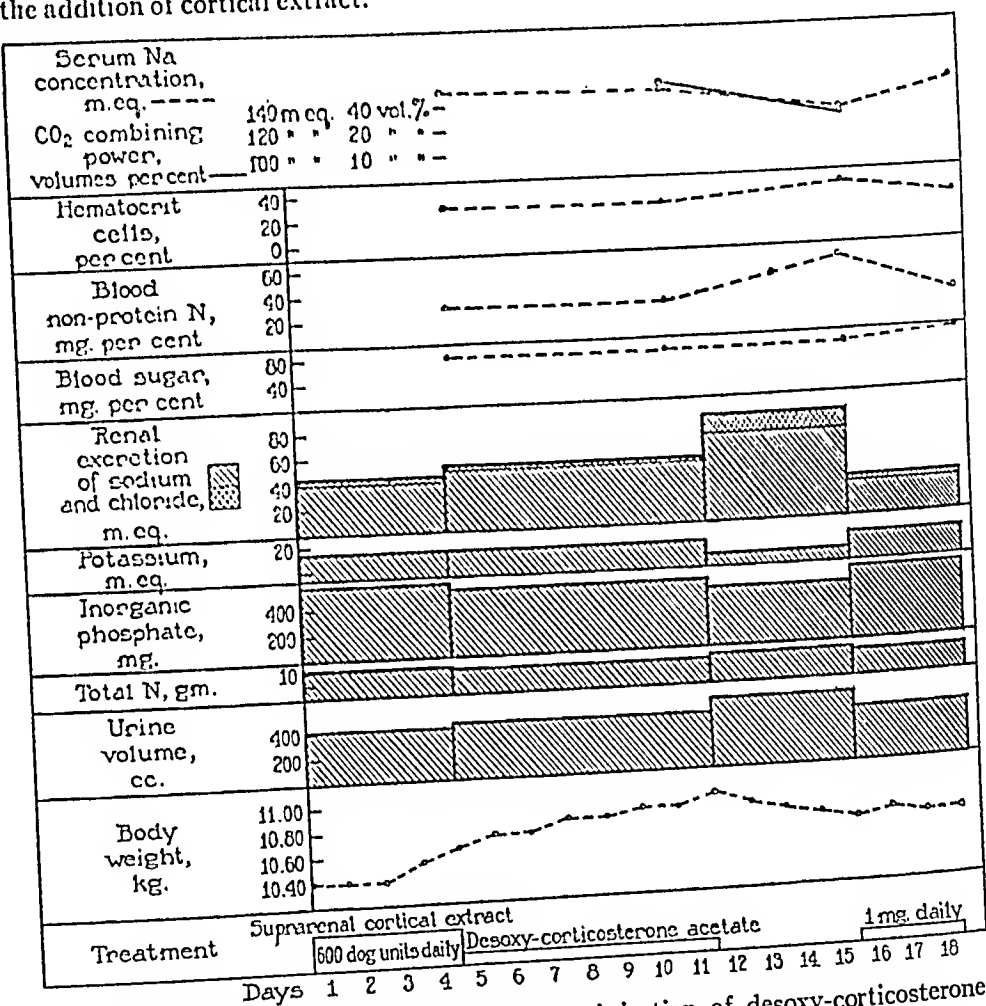

CHART 2. The effect of the subcutaneous injection of desoxy-corticosterone acetate (1 mg. daily as a single injection in oil) on the concentration of blood constituents and on the renal excretion of electrolytes in the bilaterally supra-renalectomized dog.

Dog 5 was maintained on a constant mineral and fluid intake throughout the experiment. The diet contained 62 m.eq. of sodium, 63 m.eq. of chloride, and 30 m.eq. of potassium. 1 mg. of desoxy-corticosterone acetate was taken up in 1 cc. of mazola oil.

Following this experiment the animal was maintained on suprarenal cortical extract for a period of 10 days. Extract was then discontinued and the death of the animal occurred 3 days later.

G. W. THORN, L. L. ENGEL, AND H. EISENBERG   169

It would appear from this experiment that replacement of cortical extract with the synthetic compound was complete during the short period of observation.

In studying the possible activity of compounds derived from the suprarenal cortex it is of interest to note that in the suprarenalectomized dog maintained under constant metabolic conditions the renal excretion of electrolytes furnishes an extremely sensitive index of adequate therapy (22). Within 24 hours after the reduction or withdrawal of an active preparation a marked diuresis occurs associated with a considerable loss of sodium and chloride. This change usually precedes the rise in non-protein nitrogen, and occurs while the animal still appears to be in excellent condition.

DISCUSSION

The sodium and chloride retaining effect which has been observed upon injecting suprarenal cortical extract into normal human subjects (1, 2) and normal dogs (3) appears to be due to the cortical hormone present in the extract. Of the crystalline compounds derived from the suprarenal cortex only those which have been shown to be capable of maintaining the life of suprarenalectomized animals have produced a sodium and chloride retention when injected into normal dogs in the dosage described. Compound M, which is active as measured by the Everse-de Fremery test failed to give a sodium and chloride retention when injected in quantities up to 8 mg. Desoxy-corticosterone acetate, a synthetic compound, which has been shown to be capable of maintaining suprarenalectomized dogs and rats gave a very striking sodium and chloride retaining effect when 1 mg. was injected into a normal dog. The sodium and chloride retention induced by both the extract and crystalline compounds is uniformly characteristic and does not appear to depend upon the medium used for injection, as aqueous and oil solutions have given similar responses. The striking characteristics of this response are the marked retention of sodium and chloride and the increased excretion of potassium which take place on the day of injection; and the rebound (Tables I, II, V, and VI) which occurs during the second 24 hour period. This response is not modified qualitatively by increasing the dosage of the substance injected.

Crystalline compounds which are active uniformly induce a potassium diuresis when injected into normal dogs. It appears that this

may constitute a property of the suprarenal cortical hormone although the potassium diuresis induced by the injection of suprarenal cortical extracts may be due in part to traces of epinephrin.

SUMMARY

The sodium and chloride retaining effect of suprarenal cortical extracts and of crystalline compounds derived from the suprarenal cortex parallels their effectiveness in maintaining suprarenalectomized dogs. All of the active compounds thus far studied produce a potassium diuresis when injected into normal dogs. The injection of a synthetic compound, desoxy-corticosterone acetate, produced in normal dogs a very marked sodium and chloride retention and a potassium diuresis. In a suprarenalectomized dog desoxy-corticosterone acetate was substituted successfully for suprarenal cortical extract.

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INTRAPERITONEAL AND INTRACEREBRAL ROUTES IN SERUM PROTECTION TESTS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

I. A Comparison of the Two Routes in Protection Tests

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The methods most commonly used for the detection of antiviral bodies in the sera of animals immune to the virus of equine encephalomyelitis have consisted of the injection of serum-virus mixtures intracerebrally into mice or guinea pigs. In this way it has been found that the protective power of serum was always of a low order in spite of the fact that a solid resistance was displayed by laboratory animals when tested intracerebrally or by horses convalescent from the disease.

Reports of the demonstration of antiviral substance by intracerebral technique have already been discussed (1). Experiments indicated that guinea pigs later shown to be immune to intracerebral injection of 1,000 minimal infective doses had sera which protected mice only against 1 to 10 doses or not at all. Using a method which was not quantitative, Howitt (2) found that guinea pigs immune to an intracerebral test for immunity could be shown to have protective antibodies in their sera only irregularly. Furthermore, these antibodies disappeared more rapidly than the observed immunity. Horses which have recovered from natural infection are immune to subsequent attacks but the demonstration of protective antibodies in their sera has been difficult and has usually resulted in failure (3–6). TenBroeck and Merrill (7), however, determined that in guinea pig tests antiviral bodies were revealed when serum of convalescent horses was added to low multiples of minimal cerebral infective doses of virus. They have later resorted to another method of testing with more success, namely, guinea pig pad inoculation of serum-virus mixtures.[1]

In the study of certain other viruses, it has been shown that the demonstration of protective antibodies in the sera of immune animals depends to a large extent on the route by which serum-virus mixtures

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[1] TenBroeck, C., personal communication.

are inoculated into test animals.   Variations in the degree of protection afforded by antiserum in different sites in the same species of animal are of importance in studies of the mechanism of the immune reactions in certain virus diseases.   This matter will be discussed later but the findings described in this paper provide, among other results,

TABLE I

Prior Observations in Which the Protective Power of Serum in a Serum-Virus Mixture Varied in the Same Host with the Route of Inoculation

Investigator	Virus used	Animal Injected	Route of injection resulting in protection	Route of injection resulting in less or no protection
Manteufel (8)......	Fowl pox	Chickens	Subcutaneous	On the comb
Todd (9)..........	" plague	"	Intramuscular	Intravenous
Hallauer (10).......	" "	"	"	"
Andrewes (11)......	Vaccinia	Rabbits	Intradermal	Intracerebral, intratesticular, intravenous
Craigie and Tulloch (12).............	"	"	"	Intratesticular
Fairbrother (13)....	"	"	"	Intracerebral
Sabin (14)........	"	"	"	"
Goyal (15)........	"	"	Intracerebral	Into the anterior chamber of the eye
Andrewes (11).....	Virus III	"	Intradermal	Intratesticular, intravenous
Sabin (14, 16)......	B virus	"	"	Intracerebral
Sabin (14)........	Pseudorabies	Guinea pigs	Intranasal, subcutaneous	"
Sabin (14)........	Herpes	Rabbits	Intradermal	"
Francis and Magill (17).............	Rift Valley fever	Mice	Intraperitoneal	Intranasal
Findlay (18).......	" "	"	"	"

still another example of such variation.   Table I summarizes most of the earlier reports in which differences in protective power of serum depended on the route of inoculation.

The record indicates clearly that with one possible exception, the procedure of intracerebral testing yields poorer results for the demonstration of protective power of serum than other methods.

In experimental equine encephalomyelitis there were definite indications that young mice would be infected by intraperitoneal inoculation of virus and consequently that serum protection tests might be performed by this route.

Mice less than 14 days of age have been found to be susceptible to intraperitoneal inoculation of the Western strain of this virus (19), and more recently it has been shown (20) that this animal, at the age of from 12 to 15 days, is practically invariably susceptible to both Eastern and Western strains, and even with high dilutions of virus. Although experiments by Merrill (21) with mixtures of serum and virus were designed for a purpose other than that under investigation now, and were performed in a manner different from those usually planned for the demonstration of the titre of antibody content of a serum, they leave the impression that a greater degree of protection was afforded when the intraperitoneal route rather than the intracerebral was used for inoculation of mice. In the use of serum tests for an epidemiological study, TenBroeck, Hurst, and Traub (22) stated in a footnote to a table that while most of their tests were done by intracerebral injections, they have since found the intraperitoneal route more satisfactory. No further reference to this finding was made by them.

In view of the fact that the usual intracerebral test for detection of serum antibody yielded little or no antiviral substance in spite of a high degree of resistance to virus injection (1), the question arose as to whether the weak humoral antibody content was to be regarded as absolute or whether the antiviral substance was not readily detectable by means of this method. Furthermore, one of the studies under investigation concerned a comparison of infectivity by intracerebral and intraperitoneal routes simultaneously with relative effect of serum, since prior to the work of Sabin (14), it was thought that the reason it was more difficult to demonstrate antibody by a certain route was because that route was a more sensitive indicator for the presence of virus. The mechanism governing the variations found by the two routes in the relative protective power of immune sera will be discussed, however, in a forthcoming paper.

Methods and Materials

Virus.—The Eastern strain of the virus was used in most of the experiments but the Western strain was also tried. The strains were the same as those employed in previous work in this laboratory (23) and have been maintained by intracerebral passage in mice with storage in 50 per cent buffered glycerol. In

these experiments only fresh virus was used, that is, none but the brains of mice prostrate with the disease or recently succumbed to it. Such brains were frequently kept whole in the refrigerator for several hours but were always used the same day. They were ground with alundum and enough broth to make a 20 per cent suspension; for example, two mouse brains weighing 0.8 gm. were ground with 4 cc. of broth. After centrifugation of about 2,000 R.P.M. for 2 or 3 minutes to deposit the larger particles, serial tenfold dilutions in broth were made from the supernatant. Dilutions were then 2×10^{-1}, 2×10^{-2}, etc. A fresh pipette was used for each dilution.

Sera.—Hyperimmune rabbit serum was obtained from rabbits which had received subcutaneous injections of 10 per cent suspensions of infected mouse brains in doses of 2, 4, 5, and 11 cc. at intervals of 5 to 7 days. They were bled[2] 10 days after the last dose and then at 2 to 4 day intervals. All specimens were pooled. Hyperimmune guinea pig serum consisted of pooled sera. They were derived from guinea pigs immunized with mouse brain virus followed by a test for immunity and further subcutaneous doses of active mouse brain virus.

Hyperimmune mouse serum was obtained from old mice which received intraperitoneal or intramuscular injections of mouse brain virus followed by an intracerebral test for immunity. 13 days later they were bled from the heart and the survivors bled every day or so until all were dead. These specimens were all pooled.

The serum used with the Western strain of the virus was from a rabbit that was given 2.5 cc. of a 2 per cent suspension of mouse brain virus subcutaneously and 5 cc. of a 10 per cent suspension 4 months later. It was bled for serum 9 months after that.

Five horse sera[3] were from animals in areas in New Jersey in which equine encephalomyelitis occurs, but there was no history of disease or inoculations of serum or vaccines in any of them. Eight horse sera[3] were from animals in Virginia and they had either recovered from the disease or had been in contact with known cases. Most of these sera were passed through Seitz filters to insure sterility.

All sera were stored in the refrigerator without preservative.

Serum-Virus Mixtures.—0.5 cc. of the dilution of virus was added to 0.5 cc. of undiluted serum and mixture brought about by shaking. Thus a dilution of 2×10^{-6} of virus added to an equal amount of undiluted serum gave a final dilution of virus of 10^{-6}. Virus was mixed with normal serum first and then with immune serum and the lower dilutions of virus were added to the sera before the higher. The mixtures were injected without incubation except as noted.

Mice.—All of those used were of the Rockefeller Institute albino strain. Mix-

[2] Operations on animals were performed with the aid of ether anesthesia.

[3] We wish to thank Dr. Carl TenBroeck and Dr. H. C. Givens for their cooperation and generosity in supplying these sera.

tures were injected intraperitoneally into infant mice. Their ages varied from 12 to 15 days and in one instance 16 days, but in the usual experiment all mice born on the same day were used. This particular age of mice was selected because studies of Sabin and Olitsky (20) indicated that some resistance to inoculation by the intraperitoneal route begins to appear even at 21 to 30 days. Regular results in this test depend upon taking into consideration the appearance of resistance at different ages in different mice. As will be noted, in some experiments mice born on 2 or 3 successive days but never more than 3 were employed. These young mice usually averaged in weight between 7 and 9 gm. but larger and smaller ones were encountered. Intracerebral injections were given to adult mice except as noted. Their ages are indicated. The dose by the intraperitoneal route was 0.1 cc. except as noted and the intracerebral dose was 0.03 cc. Intraperitoneal injections were made before intracerebral; immune serum-virus mixtures were injected before normal, and higher dilutions before lower. Both intraperitoneal and intracerebral inoculations of any particular dilution were made from the same tube.

Record and Estimation of Results.—The incubation period in the lower dilutions by either route was usually 2 days. Each day from then on, fewer mice developed the disease and by the 5th day practically all mice still living continued to live. Rarely one would die after that so that all animals were kept for 10 days after inoculation but most at least a week longer. Mice were considered to have developed encephalitis if they were found dead, completely prostrate, or in a state of generalized convulsions. Milder degrees of illness were observed further until such evidence developed. However, no mouse presenting definite signs of the disease has been observed by us to recover. Occasionally when there was doubt as to whether a mouse died of the disease, its brain was ground and injected into other mice to test for virus.

For convenience of designation, it was assumed that in the highest dilution in which more than half the number of the mice developed encephalitis one minimal infective dose of virus was present. In each test separate controls were included with normal serum for each route of inoculation, and results were considered only in comparison with them.

Relative Protection Obtained by Intracerebral and Intraperitoneal Methods

Hyperimmune Serum.—In the first series of experiments the relative protective power of hyperimmune sera derived from guinea pigs, mice, and rabbits was determined by the respective intraperitoneal and intracerebral injection of serum-virus mixtures. Previous intracerebral tests (1) had shown that hyperimmune serum had 10 to 100 times as much antibody, as a rule, as immune serum; that is, it protected against 10 to 100 minimal intracerebral doses of virus. The tests are summarized in Table II.

Examination of Table II shows that all of the hyperimmune sera protected against a very much larger number of minimal infective

TABLE II

Relative Protective Power of Sera in Serum-Virus Mixtures Inoculated by Intraperitoneal and Intracerebral Routes*

Experiment No.	Strain of virus	Route of injection	Age of mice (days)	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
1	Eastern	ip	16	HGP	2	1	0	0	0	0	—	—	—	10,000 Control	
		"	16	NGP	—	3	3	3	2	1	—	—	—		
		ic	30±	HGP	—	—	3	3	3	2	1	0	—		100 Control
		"	30±	NGP	—	—	—	3	3	3	3	3	1		
2	"	ip	14–15	HM	3	0	0	0	0	0	—	—	—	1,000,000 Control	
		"	14–15	NM	—	—	—	3	3	3	1	—	—		
		ic	30±	HM	—	—	—	2	3	3	—	—	—		1,000 Control
		"	30±	NM	—	—	—	—	—	3	3	2	2		
3	"	ip	14	HR	2	0	0	0	—	—	—	—	—	100,000 Control	
		"	14	NR	—	—	3	3	3	2	—	—	—		
		ic	30±	HR	—	—	—	3	3	2	0	—	—		100 Control
		"	30±	NR	—	—	—	—	—	3	2	1	0		
4	Western	ip	12	HR	3	1	0	0	—	—	—	—	—	10,000 Control	
		"	12	NR	—	—	3	3	3	1	—	—	—		
		ic	14–15	HR	—	—	—	3	3	2	0	0	0		10 Control
		"	14–15	NR	—	—	—	—	3	3	1	0	—		

— indicates not tested; HGP, hyperimmune guinea pig serum; NGP, normal guinea pig serum; HM, hyperimmune mouse serum; NM, normal mouse serum; HR, hyperimmune rabbit serum; NR, normal rabbit serum; ip, intraperitoneal; ic, intracerebral.

* Most of the experiments have been done with adult mice for the intracerebral injections and infant mice for the intraperitoneal. The reason for this was that enough young mice for all could usually not be obtained on a single day. The test recorded in Table III shows, however, that the use of adult mice for the intracerebral tests did not account for the results obtained.

doses of virus when the serum-virus mixtures were given by the intraperitoneal route to infant mice than when given by the intra-

incubation would eliminate the difference in protective power observed when unincubated mixtures were given by the two different routes.

There is an extensive record of attempts to disclose the influence of incubation on the action of antisera on viruses, although most workers agree that certain protective power can be secured without incubation being applied to serum-virus mixtures. Yet the question is important from the viewpoint of practical procedure since some viruses deteriorate at incubation temperature. Furthermore, if keeping mixtures at 37°C. could be shown to increase the action of the contained serum beyond the inactivating effect of that temperature on the virus, some evidence for an *in vitro* interaction between it and virus might be supposed to have taken place.

TABLE III

Inoculation of the Same Dose of Serum-Virus Mixtures by Intracerebral and Intraperitoneal Routes

Route of injection	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
ip	HR	3	0	0	0	0	—	—	—	—	100,000	
"	NR	—	—	—	—	3	3	2	1	—	Control	
ic	HR	—	—	3	3	2	0	—	—	—		10
"	NR	—	—	—	—	—	—	3	1	0		Control

Abbreviations as in Table II.   Eastern strain of virus.

Employing a method that was not quantitative, and the virus of equine encephalomyelitis, Howitt (5) studied the effect of incubation for varying periods. The results, however, showed no effect.   Cox and Olitsky (1) reported that with the same virus incubation of serum-virus mixtures for $2\frac{1}{2}$ hours at 37°C. increased the number of intracerebral infective units against which a serum could protect. Finally, the work of Merrill (21) with this virus indicated some interaction *in vitro* between the infective agent and the immune serum.   Table IV records the results of experiments on the effect of incubation.

The tests revealed that the protective capacity of the serum was not affected by the incubation of serum-virus mixtures when they were done in this way.   As a corollary, it is plain that the difference in degree of protective power of unincubated mixtures exhibited by the two routes was not changed by keeping them at 37°C. for $2\frac{1}{2}$ hours.

Sera from Normal Horses Derived from Epizootic Zones.—The results thus far described were obtained entirely with sera of hyperimmunized laboratory animals. Because of the striking difference in the two routes in the demonstration of protective antibody, it was now desired to determine whether the superiority of the intraperitoneal route applied to tests with horse sera and whether such procedures might be of value in epidemiological studies.

There were available for study the sera from five horses which came from districts in New Jersey where cases of equine encephalo-

TABLE IV

Effect of Incubation for 2½ Hours at 37°C. on the Protective Power of Serum When Serum-Virus Mixtures Were Given by the Intraperitoneal Route

Experiment No.	Age of mice	Incubation	Serum	Number of mice developing encephalitis of three injected									Minimal infective intra-peritoneal doses of virus against which the serum protected
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
	days												
1	14–16	None	HR	2	1	0	—	—	—	—	—	—	100,000
			NR	—	—	—	—	—	3	2	0	0	Control
		2½ hrs.	HR	1	0	0	0	0	0	—	—	—	100,000
			NR	—	—	—	—	—	2	0	0	—	Control
2	14–16	None	HR	2	1	0	—	—	—	—	—	—	1,000,000
			NR	—	—	—	—	—	3	3	2	—	Control
		2½ hrs.	HR	2	0	0	—	—	—	—	—	—	1,000,000
			NR	—	—	—	—	3	3	2	3	—	Control

Abbreviations as in Table II.   Eastern strain of virus.

myelitis have occurred. They had no clinical evidence of the disease and had not received any injections of virus, vaccines, or antiserum.

The five sera had been previously tested for antiviral substance by Dr. TenBroeck and his associates; three were found positive and two, Nos. 0815 and 0806, negative. Because of the possibility that protective capacity might be detected in the latter two by the use of the intraperitoneal technique, additional controls of broth, normal rabbit serum, or normal guinea pig serum were used. Table V shows the results of trials with these horse sera.

From Table V it will be noted that with broth or normal rabbit serum used as a control, serum 0815, previously designated as negative, protected against

10 to 100 minimal intraperitoneal infective doses and against 1 to 10 intracerebral units of virus. This result suggested that specific antibody might be present in small amounts. With normal guinea pig serum as a control, serum 0806, the other "negative" sample, protected against possibly one intraperitoneal or intra-

TABLE V

Protective Power of Horse Sera (New Jersey Series) When Serum-Virus Mixtures Were Inoculated by the Intraperitoneal and Intracerebral Routes

Experiment No.	Route of injection	Age of mice	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses of virus against which the serum protected	
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		days												
1	ip	14–15	0819	—	3	2	1	0	0	0	—	—	*	
	"	14–15	0815	—	—	—	—	2	1	1	0	—	Control?	
	"	14–15	Broth	—	—	—	—	3	3	2	1	—	Control	
	ic	25+	0819	—	—	—	2†	2	0	0	0	0		*
	"	25+	0815	—	—	—	—	—	2	2	0	0		Control?
2	ip	14–15	0692	3	2	0	1	0	—	—	—	—	*	
	"	14–15	0815	—	—	—	2	2	1	0	0	—	Control?	
	"	14–15	NR	—	—	—	—	3	2	2	2	—	Control	
	ic	21±	0692	—	—	—	3	3	3	1	—	—		*
	"	21±	0815	—	—	—	—	3	3	2	0	—		Control?
3	ip	15	0814	3	1	0	0	0	—	—	—	—	100,000 to 1,000,000	
	"	15	0806	—	—	2	2	3	2	2	0	—	Control	
	"	15	NGP	—	—	—	—	3	3	2	2	—	"	
	ic	23±	0814	—	—	—	3	3	1	0	—	—		10 to 100
	"	23±	0806	—	—	—	—	3	3	3	0	—		Control
	"	23±	NGP	—	—	—	—	—	3	3	2	1		"

Abbreviations as in Table II.
* Amount of virus against which serum protected is explained in the text.
† One died of the inoculation.

cerebral infective dose of virus. Hence serum 0806 was regarded as a more satisfactory control than serum 0815. The data of Table V were therefore evaluated on the basis of serum 0806, broth, normal rabbit and guinea pig sera as controls.

Viewed in this way, the results showed that sera 0819, 0692, and 0814, previously called positive by Dr. TenBroeck, contained protective antibodies, and 0814 protected against a larger number of infective doses of virus when given intraperitoneally than intracerebrally. The latter sample rendered from 10 to 100 units of virus non-infective by the intracerebral test and 100,000 to 1,000,000 by the intraperitoneal method.

The supply of serum 0806 was soon exhausted; a horse serum was therefore sought which showed no protective power by this intraperitoneal technique for use as control in further experiments.

Horse M 33 had been immunized with meningococci and bled for serum on Oct. 7, 1919. This serum was sealed and stored in the refrigerator in this laboratory until Feb. 7, 1938. On the latter date a portion of it was passed through a Seitz filter. An electrometric determination of pH was 7.8 and cultures yielded no growth of bacteria so that it was believed not to have essentially deteriorated.

The serum M 33 was then tested in comparison with normal guinea pig serum as recorded in Experiment 1 of Table VI. The outcome was a difference in titre of only one minimal intraperitoneal infective dose (as with serum 0806) and since this was not significant in respect to the number of animals employed with each dilution, it was decided to use M 33 as a control for further tests with horse sera.

Sera from Horses Recovered from, or Exposed by Contact to Equine Encephalomyelitis.—The next series of tests were performed on sera obtained from four horses that had shown clinical signs and recovered from equine encephalomyelitis, and four others known to have been in contact with one to four horses having signs of the malady. All the animals were from epizootic areas in Virginia and the sera were collected from 6 months to 4 years after recovery from or contact with the disease. Six separate experiments were undertaken and these are recorded in Table VI.

In Table VI it can be seen that sera 1 and 5 protected against a larger amount of virus intraperitoneally than intracerebrally; in the instance of serum 1, 100,000 times as much. Experiments 3, 4, and 5 were not planned to determine the amount of virus against which a serum protected but to show whether the existence of antibody could be detected with a set dose, so as to give a practical aspect to the intraperitoneal test (Experiment 4). In this way, every one of the sera of horses known to have recovered from equine encephalomyelitis showed

TABLE VI

Protective Power of Horse Sera with Horse Serum M 33 as Control

Experiment No.	Route of injection	Age of mice	Serum	Clinical history of horse	Number of mice developing encephalitis of three injected								Minimal infective doses of virus against which the serum protected	
		days			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Intraperitoneal doses	Intracerebral doses
1	ip	15	M 33*	See text	—	—	—	3	3	3	1	—	1	
	"	15	NGP	Control	—	—	—	—	3	3	2	0	Control	
2	"	13	1*	Recovered Sept., 1933	3	1	—	0	0	—	—	—	100,000	
	"	13	M 33*	Control	—	—	—	—	3	3	2	0	Control	
	ic	20	1*	Recovered Sept., 1933	—	—	—	3	3	2	0	0		1
	"	20	M 33*	Control	—	—	—	—	3	3	3	1		Control
3	ip	12–13	2	Exposed to No. 1 Sept., 1933	—	—	—	4†	4†	4†	—	—	0	
	"	12–13	3*	Recovered Sept., 1934	—	—	—	0†	0†	0†	—	—	At least 100	
	"	12–13	4	Recovered Sept., 1934	—	—	—	0†	0†	0†	—	—	At least 100	
	"	12	M 33*	Control	—	—	—	4†	4†	4†	—	—	Control	
4 (use of single test dose)	"	12–13	5*	Exposed to No. 4 Sept., 1934	—	—	2†	—	—	—	—	—	?	
	"	12–13	6*	Recovered Aug., 1937	—	—	0†	—	—	—	—	—	At least 100	
	"	12–13	8*	Exposed to 4 sick horses Aug., 1937	—	—	4†	—	—	—	—	—	?	
	"	12–13	9*	Exposed to 2 sick horses Aug., 1937	—	—	3†	—	—	—	—	—	?	
	"	12–13	M 33*	Control	—	—	—	—	—	3	2	1	Control	

	No. of mice		History										Titer
5	"	12	0815	See Table V	—	—	3	3	1	3	—	—	—	10
	"	12	5*	Exposed to No. 4 Sept., 1934	3	—	0	0	0	3	—	—	—	1,000
	"	12	8*	Exposed to 4 sick horses Aug., 1937	—	—	3	3	3	—	—	—	—	Less than 10
	"	12	9*	Exposed to 2 sick horses Aug., 1937	—	—	3	3	2	—	—	—	—	Less than 10
	"	12	M 33*	Control	—	—	—	3	3	2	—	—	—	Control
6	"	12	5*	Exposed to No. 4 Sept., 1934	3	3	1	1	—	3	—	—	—	100
	"	12	M 33*	Control	—	—	—	3	3	1	—	—	—	Control
	ic	Adult	5*	Exposed to No. 4 Sept., 1934	—	—	1	2	0	0	—	—	—	1
	"		M 33*	Control	—	—	—	3	3	3	—	—	—	Control

* Filtered through a Seitz filter.
† Four mice injected.

power to protect against the virus. Furthermore, the serum of one horse which did not have any illness that was recognized but had been in contact with the disease also gave evidence of specific antibody.

The results of the tests with horse sera show the intraperitoneal route to be more sensitive than the intracerebral (0814, 1, and 5) and also that the intraperitoneal technique would probably be a valuable tool for epidemiological studies since all of the sera of horses known to have had the disease gave strongly positive reactions (1, 3, 4, and 6) and sera of others (four of nine) not known to have shown clinical signs but which have been in contact with the disease also contained measurable, definite antibody (0819, 0692, 0814, and 5).

DISCUSSION

The method ordinarily employed heretofore for the recognition and measurement of humoral antibody in equine encephalomyelitis has consisted of the injection of serum-virus mixtures into the brains of mice. The present experiments show that the intraperitoneal route is more sensitive for this purpose. The basis for this is to be found in the uniform susceptibility of 12 to 15 day old mice to the intraperitoneal injection of the virus (20). In most instances there is only a tenfold or no difference between intracerebral and intraperitoneal titers; 12 to 15 day old mice are approximately equally susceptible to inoculation by the two routes.

The intraperitoneal procedure has been shown to be applicable not only to the sera of laboratory animals immunized with active virus but also to the sera of horses naturally infected, or of those exposed by contact to the disease. It should be of value not only because of its ability to detect antibody to a much higher degree than the intracerebral method, but also, in view of the sensitiveness of the test, because of its capacity to indicate negative findings with greater assurance that antibody is not at all present.

The results of the application of this test to horse sera do not permit general conclusions because of the small number of specimens examined. Nevertheless, they furnish some indication that horses recovered from the disease have serum antibodies regularly and that these may persist for at least 4 years. In addition, antibodies may be found in the sera of horses that have shown no signs of the disease but that

live on farms where the infection has been prevalent, while others from such farms may be negative. That the sera of horses not having clinically apparent disease may contain antiviral substance has already been found by TenBroeck, Hurst, and Traub (22) and confirmed by Giltner and Shahan (6). A more extensive investigation on larger numbers of animals exposed by contact is necessary before one can say whether the intraperitoneal method can disclose a higher percentage of positive reactions for antibody than the intracerebral or other methods.

In prior reports in which animals were described as solidly immune to equine encephalomyelitis, it has been stated that this immunity was associated with a minimal amount of protective antibody in the serum. The present experiments show that perhaps the discrepancy between the amounts of immunity and antibody can be explained by the demonstration of large amounts of antibody by the method of intraperitoneal test.

Certain aspects of the mechanism underlying the phenomenon of the superiority of antibody detection by the intraperitoneal test will be discussed in a forthcoming paper. For the present, some remarks may be made with regard to the reaction of immune serum and virus *in vitro*.

It has been mentioned that the work of Merrill (21) indicated some kind of interaction *in vitro* between this virus and serum. He concluded from his experiments that combination between virus and antibody had occurred *in vitro* probably resulting in aggregation of virus particles. It should be stated that our experiments do not give evidence as to whether there is combination in the test tube. They do demonstrate, however, that in the dilutions which show protection by the intraperitoneal route and not by the intracerebral, the infectious activity has not been abolished *in vitro* by the immune serum; in other words, that the immune serum is not directly virucidal by the intraperitoneal route. This is evident from the fact that material taken from a given tube may not give rise to infection if injected intraperitoneally but will if inoculated intracerebrally. If antibody has combined with virus in such tubes, the combination must be dissociable when in contact with certain tissues, inasmuch as protection may or may not occur, depending on the tissue into which the serum-virus mixture is injected. That variation in protective power of

antiviral serum according to route of inoculation indicates that the consummation of the immune reaction is not based on direct inactivating effect, has been suggested before by several workers among whom may be mentioned Andrewes (11), Sabin (14), and Francis and Magill (17).

Finally to be stressed in this discussion is the point that the behavior of serum-virus mixtures, when injected by different routes, is not the result of the greater capacity of one route to detect unneutralized virus, a fact first demonstrated by similar quantitative, comparative titrations for vaccinia, herpes, B virus, and pseudorabies viruses (Sabin, 14).

SUMMARY AND CONCLUSIONS

Young (12 to 15 day old) mice are approximately as susceptible to the virus of equine encephalomyelitis, Eastern or Western strain, when it is given intraperitoneally as are adult mice when the virus is injected intracerebrally. With this susceptibility by the intraperitoneal route as a basis, the injection of immune serum-virus mixtures intraperitoneally was found to result in protection in dilutions which give rise to infection after intracerebral inoculation.

The difference of protective power by the two indicated routes was shown not to depend on the amount of inoculum nor on the age of the intracerebrally injected mice. Incubation at 37°C. for $2\frac{1}{2}$ hours neither increases nor diminishes the protective action of immune serum when the intraperitoneal method is employed.

The phenomenon of selective protection in different tissues is elicited by the sera of hyperimmunized mice, guinea pigs, and rabbits and by sera derived from horses infected with the disease in nature or exposed to it by contact. Of four horses recovered from the malady, all showed antibody in their sera; of others exposed by contact, four of ni animals revealed antiviral bodies, when the intraperitoneal techniq was employed. These tests on horse sera have pointed to the potential value of this procedure for epidemiological studies.

Finally, the reaction itself has significance through its bearing the mechanism of immunity.

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PRODUCTION OF EXPERIMENTAL OSTEOMYELITIS IN RABBITS BY INTRAVENOUS INJECTION OF STAPHYLOCOCCUS AUREUS

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PLATES 4 AND 5

In the course of experiments designed to compare the virulence of different strains of staphylococcus in rabbits, it was observed that two of the animals developed well characterized bone lesions following the intravenous injection of a certain strain of *Staphylococcus aureus*.

The literature concerning the production of staphylococcal osteomyelitis in experimental animals reports many conflicting results; most workers failed to produce the condition unless elaborate procedures were resorted to in the hope of localizing the staphylococci in the bones. As early as 1885, however, Rodet (1) had succeeded in producing bone abscesses in rabbits by the intravenous injection of cultures of *Staphylococcus aureus,* and even before that time other attempts had been made involving the preliminary production of local trauma by fracture or contusion of the bones. Yet in 1922 Starr (2) stated that he found "great difficulty in producing osteomyelitis in the dog, even with staphylococci or streptococci of known virulence in man," and was finally induced to use the laborious technique of ligating the appendix and its mesentery, after which he injected the mixed organisms from the peritoneal exudate into a nutrient artery or into a traumatized metaphysis; by this means he succeeded in producing acute osteomyelitis in growing dogs, but in every case the animal died within 48 hours, so that the late changes of bone destruction and sequestration were never seen. Various other more or less elaborate techniques have also been used; thus Kuwahata (3) and Takahashi (4) first fed their animals on a diet deficient in ascorbic acid, while Haldeman (5) implanted cultures into holes drilled into the metaphysis. Yet as late as 1935 Baudet and Cahuzac (6) injected staphylococci into rabbits by the intravenous, subperiosteal, and intrametaphyseal routes, and concluded that it was impossible to produce a true osteomyelitis in experimental animals. Shioda (7) on the other hand, obtained consistent results by the intravenous injection of a strain of staphylococcus isolated from a case of osteomyelitis; his publication,

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however, is more concerned with the pathology of the condition than with the problem of its production.

The present paper records observations on osteomyelitis which appeared in rabbits following inoculation with a strain of *Staphylococcus aureus*. They seem the more worth recording in view of the contradictory results which have been obtained by other workers.

Methods and Materials

1. Description of the Strain.—The strain of staphylococcus (OH) used in this study was isolated from the lungs in a fatal case of bronchopneumonia admitted to this hospital 19 years ago. During these 19 years the culture has been transferred once a month on meat infusion agar; it was not possible to find any record of animal passage. The strain belongs to the *aureus* type, although it forms only very small amounts of the orange pigment. No effort has been made to place it in any of the recent schemes of classification of staphylococci. The culture is strongly hemolytic and forms an active coagulase. Throughout the present work it has been carried in meat infusion broth.

2. Experimental Procedure.—Rabbits were infected intravenously with a small dose of a broth culture, and were then observed daily for the development of any swelling, tenderness, or loss of function of any of the limbs. In 3 of the experiments x-ray photographs were taken of the animals before death and also of any inflamed bones after removal from the body at autopsy. In many of the cases, particularly where no obvious suppuration was visible, smears of the bone marrow were stained and examined microscopically for the presence of staphylococci and for evidence of an inflammatory reaction.

The autopsies were carried out with the primary purpose of detecting any bone lesions; particular attention, however, was also paid to the condition of the joints, in the hope of deciding whether lesions in the metaphyses of the long bones were due to a primary osteomyelitis or to a spread from a primary purulent arthritis. In all cases in which the pus was cultured on blood agar plates, staphylococci were recovered in pure culture from the bone abscesses. Any bones presenting evidence of inflammation, or of a periostitis, were removed from the body at autopsy; the majority of the soft tissue around the bone was then cut away, and the bone lesion more clearly defined.

Virulence of the Strain for Rabbits

Since the culture (OH) had been maintained on nutrient agar over a period of 19 years, an effort was made to raise its virulence by animal passage.

The culture was injected into normal rabbits by the intravenous route. In its original condition the culture failed to cause obvious signs of infection when used

in amounts of 0.5 cc. or less; on the other hand 2 rabbits inoculated with 1 cc. died within 24 hours and a pure culture was recovered from the heart blood. This passage strain was injected into new rabbits; one of the animals inoculated with 0.5 cc. culture under the conditions described in Experiment 1 became pyrexial and was killed after 17 days. A pure culture of *Staphylococcus aureus* was recovered from a bone abscess found at autopsy and this culture was designated as OH 172.

Culture OH 172 has been inoculated into a number of normal young and adult rabbits. In all cases 0.1 cc. was found to kill the animals within 48 hours after injection, whereas 0.01 and 0.001 cc. caused the development of a chronic form of infection associated with pyrexia.

It is evident, therefore, that the virulence of the strain for the rabbit increased markedly as a result of animal passage.

The Production of Experimental Osteomyelitis in Rabbits

The following experiment had been planned in order to test the virulence of this particular strain of staphylococcus. The infection became subacute in 2 of the infected rabbits; they developed swellings in the regions of the limbs. As it was suspected that the underlying cause might be an osteomyelitis, autopsies were carried out.

Experiment 1.—4 rabbits were inoculated with the heart blood culture of the OH strain mentioned above; 2 received 0.1 cc. (rabbits 1 and 2) and 2 received 0.01 cc. (rabbits 3 and 4).

Rabbit 1 died 4 days later; culture of the heart blood gave a mixed growth of staphylococci and Gram-negative cocci. This culture was discarded. The other 3 rabbits remained apparently normal, and were reinoculated with larger amounts of the same culture, rabbit 2 receiving 1 cc. of culture and rabbits 3 and 4, 0.5 cc. each. Rabbit 2 developed a palpable fluctuating swelling of the left knee, and was killed on the 20th day after reinfection. Rabbit 3 developed a massive, fluctuating swelling in the region of the left shoulder joint and was killed on the 17th day. Rabbit 4 died on the 2nd day after reinfection; no autopsy was performed.

Autopsy Findings.—Rabbit 2. Left knee joint distended in the gross with yellow, viscous pus. Extensive bone destruction of lateral and posterior surfaces of lateral condyle of left femur. Area of softening and hyperemia also present on lateral aspect of upper end of tibial portion of left tibiofibula. Right knee joint contained small amount of seropurulent fluid; marked softening and hyperemia, with subperiosteal bleeding points, of medial aspect of upper end of right tibia; bone very readily penetrated by a blunt probe; medullary cavity contained viscous semipurulent material. Bone marrow smear from left femur showed large

numbers of staphylococci, many of which were undergoing destruction, many polymorphonuclear leucocytes, and extensive phagocytosis. Bone marrow smear from left tibia showed very few staphylococci and large numbers of polymorphonuclear leucocytes.

Rabbit 3. Large abscess containing yellowish white, inspissated pus surrounding upper end of left humerus and left shoulder joint. Humero-acromial joint disorganized, and joint cavity full of pus. Extensive bone destruction of head and neck of left humerus, and replacement of the marrow with pus. A pure culture of *Staphylococcus aureus* was recovered from this abscess; as already mentioned, this culture was designated as OH 172, and subsequent experiments were carried out with this strain. Moderate bone destruction of glenoid cavity of scapula. Encapsulated subperiosteal collection of pus present on the outer side of twelfth left rib, at about two-thirds of the way along its length from the costovertebral joint. Bone marrow smear from humerus showed large numbers of staphylococci and pus cells; moderate amount of phagocytosis also present.

In view of the positive results obtained in 2 of the animals in Experiment 1, 3 more rabbits were infected.

Experiment 2.—3 normal adult rabbits (Nos. 5, 6, and 7) were inoculated with 0.1 cc. each of a culture of OH 172. All 3 rabbits died within 48 hours. On account of the acute course of the infection autopsies were not performed.

It is apparent that, in order to obtain convincing evidence of bone inflammation in rabbits inoculated with staphylococci, it is essential to arrange the experimental conditions so as to obtain a relatively prolonged course of the infection. To achieve this end an attempt was made to establish in rabbits a low grade of immunity, prior to infection, by immunizing them with suspensions of killed staphylococci. The results described above, illustrating the virulence of the culture, have shown that if the infective dose be high enough, the rabbits die within a very few days; care was therefore taken to avoid using infective doses large enough to cause a fulminating course of the infection.

Experiment 3.—6 normal adult rabbits (Nos. 8 to 13) weighing about 2000 gm. each, were immunized with the following materials.

(*a*) 2 cc. of formol were added to 150 cc. of a broth culture of OH 172; the bacterial suspension was allowed to stand at room temperature for 48 hours. It was then centrifuged and resuspended in 15 cc. of M/20 phosphate buffer at pH 7.0.

(*b*) 200 cc. of a broth culture OH 172 were brought to pH 4.3 with acetic acid, and allowed to stand at this pH for 5 days at 37°C. The cells were then centrifuged and resuspended in 20 cc. saline.

(c) The cells from 150 cc. of a broth culture OH 172 were centrifuged, re-suspended in 15 cc. saline, and then heated at 80°C. for 30 minutes.

The animals received 0.5 cc. of the bacterial suspension by the intravenous route on 3 consecutive days. Rabbits 8 and 9 received the formolized suspension; rabbits 10 and 11 the cells killed with acetic acid; and rabbits 12 and 13 the heat-killed cells. All 6 rabbits were inoculated 1 week later with 0.1 cc. of a broth culture of OH 172. Rabbits 8, 10, 11, and 12 became very sick; rabbit 12 died on the 8th day, rabbits 10 and 11 on the 9th day, and rabbit 8 on the 10th day; in none of them was it possible to obtain clinical evidence of any localized inflammation. Rabbits 9 and 13 developed a chronic condition; x-ray photographs were taken of their hind limbs on the 24th day.

X-Ray Photographs of Rabbit 9.—Large area of rarefaction present in the metaphysis at upper end of left tibia, visible in both anteroposterior and lateral views.

X-Ray Photographs of Rabbit 13.—Upper end of right fibula thickened, with a rounded area of rarefaction present in it. Localized area of rarefaction also present in compact bone at medial aspect of upper end of left tibia.

By the 29th day rabbit 13 was showing marked loss of use of both hind limbs. Rabbit 9, however, appeared no worse, so 0.5 cc. of a broth culture of the same staphylococcal strain was injected intravenously. Both animals were killed on the 33rd day. Rabbits 10 and 12 showed evidence of bone inflammation at autopsy.

Autopsy Findings.—Rabbit 8. Large collection of pus overlying upper end of right fibula; on dissection an area of bone destruction was discovered at lateral aspect of upper end of bone, with frank pus in medullary cavity. Large abscess also present in lower end of left femur; left knee joint contained a serous exudate. Localized collection of pus attached to 9th right rib, with bony destruction of wall of rib and pus in medullary cavity. Bone marrow smears from lesions in fibula and femur both showed presence of large numbers of staphylococci and pus cells.

Rabbit 9. Marked roughening and absorption of bone in region of upper metaphysis of left tibia, with multiple bleeding points on raising the periosteum. Bone easily perforated by blunt probe. Area of irregular subperiosteal new bone also present on shaft of left tibia. X-ray photograph of left tibia after death showed marked rarefaction of head of the bone. Bone marrow smear showed presence of Gram-positive cocci, a few polymorphonuclear leucocytes, and some lymphocytes.

Rabbit 11. Abscess present in soft tissues overlying anterolateral aspect of right tibia. Underlying bone presented an area of spongy new bone deposition.

Rabbit 13. Marked irregular thickening of upper half of right fibula, with an abscess eroding through posterior wall at upper end. Small, localized bone abscess at upper end of medial border of left tibia; posterior surface of upper end of the bone also showed marked softening and hyperemia. X-ray photograph

of the bones after death showed irregular thickening of upper end of right fibula with several rounded areas of rarefaction; mottling of upper end of right tibia with small area of rarefaction in compact bone along medial aspect. Bone marrow smear from fibula showed presence of large numbers of staphylococci and pus cells; smear from left tibia showed many pus cells, but no cocci.

X-ray photographs of the bone lesions in rabbits 9 and 13 are shown in the accompanying plate (Figs. 1 to 7).

The results of the previous experiments show that under suitable conditions it is possible to produce inflammation of the bones in normal adult rabbits by the mere intravenous injection of staphylococci. In view of the fact that in human beings staphylococcal osteomyelitis is much more common in young individuals than in adults, it was of interest to apply the experimental procedure used in Experiment 3 to younger rabbits.

Experiment 4.—9 young rabbits (Nos. 14 to 22), about 2 months old, were immunized on 3 consecutive days by the intravenous injection of 0.5 cc. of a formolized suspension of staphylococci (prepared as in Experiment 3). The 9 rabbits were inoculated 7 days later with 0.1 cc. of a culture of OH 172. Rabbits 15, 16, 18, and 22 were found dead on the morning of the 2nd day; autopsies were not done, as it was considered too early to show any macroscopic bone changes. The other 5 rabbits died at various times within the next 8 days; all of them showed marked weakness and loss of appetite, but it was not possible to detect in them any definite localizing signs suggestive of bone involvement.

Autopsy Findings.—Rabbit 14. Suppurative arthritis of right knee joint. No visible bone suppuration or inflammation. Right femur and right tibiofibula removed for x-ray examination. X-ray photographs of femur showed mottling and trabeculation of upper end of bone, with a circumscribed area suggestive of advanced bony absorption in the neck. Right tibia showed no abnormality. Bone marrow smear from neck of right femoral head showed many polymorphonuclear leucocytes, a few Gram-positive cocci and many Gram-negative cocci at varying stages of autolysis; extensive phagocytosis.

Rabbit 17. Marked hyperemia of soft tissues overlying upper end of right tibiofibula. On dissection, right fibula seen to be hyperemic and roughened, with multiple subperiosteal bleeding points; no suppuration or advanced bone destruction. Bone marrow smear from fibula showed many Gram-positive cocci and pus cells.

Rabbit 19. No evidence of bone inflammation. Slight suppurative arthritis of right ankle joint.

Rabbit 20. Suppuration and bone destruction in left scapula, near the vertebral border. Also in upper end of right tibia at its posterior aspect. Suppura-

tive arthritis of right elbow joint, with bone abscess in lateral border of olecranon process of right ulna. X-ray photographs of isolated bones showed extensive bone destruction along vertebral border of left scapula; area of rarefaction at lateral aspect of upper end of left tibiofibula. On dissecting away the soft tissues surrounding the lower end of the left tibiofibula, an area of destruction of the wall of the bone was discovered corresponding to the lesion in the x-ray picture, the cavity being filled with yellowish white pus. Bone marrow smears from scapula, right tibia, and left tibiofibula all showed the presence of large numbers of staphylococci and some pus cells.

Rabbit 21. Inflammation and hyperemia of soft tissues overlying posterior aspect of lower end of left femur; subjacent bone hyperemic and roughened, with bleeding points on raising the periosteum; bone readily penetrated by blunt probe. Bone marrow smear showed large numbers of staphylococci and polymorphonuclear leucocytes; some phagocytosis and some leucocytes undergoing destruction.

The results of Experiment 4 show that culture OH 172 is so virulent for young rabbits that most of them survived the injection of 0.1 cc. of the culture for only a few days, even though they had received a course of immunization prior to infection. In the hope of prolonging the course of the disease, the infective dose was therefore cut down to 0.01 cc.

Experiment 5.—6 young rabbits (Nos. 23 to 28) were given 3 courses of immunization by a method similar to that used in the preceding experiment. 7 days after the last immunizing dose each of them received 0.01 cc. of a broth culture of OH 172. On the following day all the animals became pyrexial; daily rectal temperatures were taken throughout the course of the disease:

Rectal Temperatures

Day............	1	2	3	4	6	7	8	9	10	11	13
Rabbit 23	106.1	106.2	106.7	106.1	105.5	104.7	102.8	98.6	102.9	102.1	Killed
24	106.2	106.3	105.9	105.4	105.9	105.5	104.7	105.1	—	99.8	Killed
25	106.4	105.7	105.8	105.1	105.1	105.1	104.6	104.4	Killed		
26	106.4	106.0	106.3	105.4	105.3	105.3	104.4	103.4	Died		
27	105.8	106.0	106.6	105.1	105.1	103.5	102.6	101.6	—	Killed	
28	105.6	106.6	106.4	105.2	105.6	105.3	105.1	102.6	Killed		

All 6 rabbits became very sick; palpable swellings became noticeable in the limbs; and x-ray photographs were taken of the animals on the 7th day.

X-Ray Photographs on the 7th day.—Rabbit 23. Area suggestive of rarefaction at lower end of left femur.

Rabbit 24. Marked evidence of rarefaction at lower end of right femur, with appearance suggestive of sequestration. Suggestion of bony absorption at upper end of right tibia.

Rabbit 25. No bone abnormality observed.

Rabbit 26. Area of rarefaction with suggestion of sequestration at upper end of left tibia.

Rabbit 27. No bone abnormality observed.

Rabbit 28. Area of rarefaction at head of right femur, with suggestion of bone destruction at lower end of the bone just above the condyles.

Rabbits 23 and 24 had x-ray pictures taken again on the 13th day and then were killed.

X-Ray Photographs on 13th day.—Rabbit 23. Marked rarefaction at lower end of left femur; small rounded area of rarefaction also present at upper end of left tibia.

Rabbit 24. Large area of rarefaction with suggestion of sequestration at lower end of right femur. Similar appearance present at upper end of right tibia. Rarefaction also visible on lesser trochanter of right femur and at medial aspect of lower end of left femur. Bone erosion also apparent at head of left fibula.

Autopsy Findings.—Rabbit 23. Abscess with extensive bone destruction at lower end of left femur, situated on the posteromedial aspect of medial condyle. Suppuration and bone destruction also present at posterior aspect of upper end of left tibia and at upper end of right tibia.

Rabbit 24. Abscesses with varying degrees of bone destruction found at the following sites: lateral aspect of upper end of left fibula; medial aspect of lower end of left tibia; medial aspect of upper end of right tibia; anterior aspect of lower end of right femur; anterior aspect of lesser trochanter of right femur; medial aspect of lower end of left femur; lateral aspect of greater trochanter of left femur; olecranon process of right ulna; posterior aspect of upper end of left humerus; 7th right rib; vertebral border of right scapula.

Rabbit 25. Abscesses with varying degrees of bone destruction present in greater trochanter of right femur, upper end of right fibula, upper end of left tibia (with sequestrum), medial aspect of head of left humerus, upper end of right tibia, lower end of left tibia, and lower end of right femur. Right knee joint contained a serous exudate, but no pus. Bone marrow smears from right fibula and left tibia showed many polymorphonuclear leucocytes and much phagocytosis; a few extracellular Gram-positive staphylococci.

Rabbit 26. Area of spongy hyperemic bone, with multiple subperiosteal bleeding points, at lateral aspect of upper end of left tibia; no suppuration visible on surface of bone. Small abscess over anterior border of right tibia, with subjacent area of bony roughening with some new bone deposition. Bone marrow smear from left tibia showed marked infiltration with polymorphonuclear leucocytes; some evidence of phagocytosis; no extracellular cocci seen.

Rabbit 27. Small, localized abscess over lesser trochanter of left femur; area of bone destruction in subjacent bone with a separated sequestrum. Abscesses with bone destruction also present on medial aspect of upper end of left tibia, medial and inferior aspect of head of left humerus and 10th right rib.

Rabbit 28. Large abscess with bone destruction at posterior aspect of medial condyle of right femur. Suppuration and bone destruction also present at head of right femur. Suppurative arthritis of right hip joint. Bone marrow smear from lower end of femur showed large numbers of staphylococci and pus cells.

The X-ray photographs of some of the bone lesions in Rabbits 24 and 25 are shown in the accompanying plate (Figs. 8 to 15).

Experiment 6.—The 3 rabbits (Nos. 29 to 31) in this experiment were used for a virulence test of culture OH 172. Since two of them developed bone lesions they have been included in the present series.

Rabbit 29 was injected with 0.0001 cc. of a broth culture of OH 172, rabbit 30 with 0.001 cc., and rabbit 31 with 0.01 cc. All 3 rabbits became pyrexial. Rabbit 29 developed a gross swelling in the region of the left knee. Rabbit 30 progressed without the development of any physical signs. Rabbit 31 developed a massive, tense swelling of the right fore leg, and was killed on the 9th day. The other 2 animals were killed after x-ray photographs had been taken on the 19th day.

X-Ray Photographs on 19th Day.—Rabbit 29. Rarefaction with loss of outline of medial condyle of left femur; some blurring of epiphyseal line.

Rabbit 30. No bone abnormality observed.

Autopsy Findings.—Rabbit 29. Suppurative arthritis of left knee joint. Extensive bone destruction of lower end of left femur with erosion through the epiphysis and articular surface. Articular surface of upper end of left tibia also eroded, but lesion did not progress deeply into the bone, and appeared to be secondary to the advanced suppuration in the joint cavity.

Rabbit 30. No evidence of bone inflammation.

Rabbit 31. Bone abscess with sequestration at posterior aspect of lower end of right tibia. Suppuration and bony destruction present at lower end of left humerus, with suppurative arthritis of the elbow joint. Small abscess with area of bone erosion also present at upper end of left humerus. Very extensive bony destruction and suppuration in the right humerus, affecting almost the entire thickness of the bone, and bursting through and eroding a large area of the lateral aspect of the bone. Subperiosteal collection of pus attached to 3rd right rib; on dissection it was found to have tracked out from the rib, the medullary cavity containing thick white pus.

A summary of the lesions found in the autopsies described above, together with the length of the disease process in days, is given in Table I. Table II shows the number and localization of the lesions found. Photograph of bone abscesses in a humerus and in a rib is shown in Text-fig. 1.

TABLE I

Summary of Lesions

Rabbit	Length of disease process	Number and distribution of bone lesions
	days	
1	4	Nil
2	20	(1) Lower end left femur (2) Upper end left tibia (3) Upper end right tibia
3	17	(1) Head left humerus (smear) (2) 12th left rib
4	1	Nil
5	1-2	Nil
6	1-2	Nil
7	1-2	Nil
8	10	(1) Upper end right fibula (2) Lower end left femur (3) 9th right rib
9	33	Upper end left tibia
10	9	Nil
11	9	Right tibia (subperiosteal osteogenesis)
12	8	Nil
13	33	(1) Upper end right fibula (2) Upper end left tibia
14	4	Neck right femur
15	2	Nil
16	2	Nil
17	2	Upper end right fibula
18	2	Nil
19	5	Nil
20	8	(1) Vertebral border left scapula (2) Upper end right tibia (3) Olecranon process right ulna (4) Lower end left tibiofibula

TABLE I—*Concluded*

Rabbit	Length of disease process	Number and distribution of bone lesions
	days	
21	3	Lower end left femur
22	2	Nil
23	13	(1) Lower end left femur (2) Upper end left tibia (3) Upper end right tibia
24	13	(1) Upper end left fibula (2) Lower end left tibia (3) Upper end right tibia (4) Lower end right femur (5) Upper end right femur (6) Lower end left femur (7) Upper end left femur (8) Olecranon process right ulna (9) Upper end left humerus (10) 7th right rib (11) Right scapula
25	10	(1) Lower end right femur (2) Greater trochanter right femur (3) Upper end right fibula (4) Upper end right tibia (5) Head left humerus (6) Upper end left tibia (7) Lower end left tibia
26	10	(1) Upper end left tibia (2) Upper end right tibia
27	11	(1) Lesser trochanter left femur (2) Upper end left tibia (3) Head left humerus (4) 10th right rib
28	10	(1) Lower end right femur (2) Head right femur
29	19	(1) Lower end left femur (2) Upper end left tibia
30	19	Nil
31	9	(1) Lower end right tibia (2) Lower end left humerus (3) Upper end left humerus (4) Upper end right humerus (5) 3rd right rib

TABLE II

Number and Localization of Lesions

Total number of animals used	31
Number of animals surviving immediate toxic effects of injection	22
Number of animals with bone lesions	18
Total number of lesions	55
Relative frequency of site of lesions:	
Upper end of tibia	14
Lower end of femur	9
Upper end of femur	6
Upper end of humerus	6
Fibula	5
Ribs	5
Lower end of tibia	4
Scapula	2
Ulna	2
Lower end of humerus	1
Shaft of tibia	1

TEXT-FIG. 1. Photograph of bone abscesses after removal of bones from the body and after the soft tissues had been dissected away. The humerus shows an area of bone destruction on its anterior aspect with pus lying in the eroded area; the rib shows an encapsulated collection of pus issuing from its external surface.

DISCUSSION

The object of the present study is to describe the conditions under which a certain strain of staphylococcus (OH) causes the development of bone lesions in rabbits; the study is not aimed at an analysis of the pathology of staphylococcal osteomyelitis.

The strain of staphylococcus used in this work had been cultivated on meat infusion agar over a period of 19 years; it proved to be of a very low degree of virulence for the rabbit. Following animal passage, however, and especially after recovery of the strain from a bone abscess discovered in one of the rabbits infected with a large dose, the virulence increased so markedly that 0.1 cc. of the culture was sufficient to kill adult animals in 24 to 38 hours.

Such a fulminating course of the infection is not favorable for the development of easily recognizable bone lesions, and it seemed likely that the production in the rabbits of a low degree of antistaphylococcal immunity (antibacterial or antitoxic) would help towards the production of a chronic type of infection. The data, however, are not sufficient to demonstrate that immunization was of great significance in determining the development of bone lesions, and it is possible that satisfactory results would have been obtained by the mere injection of small infective doses into normal rabbits (Experiment 6). On the other hand, immunization did appear to stabilize the animals against the early lethal effect of larger doses of staphylococci, and thereby facilitated the production of progressive bone inflammation through a wider range of infective doses.

In any case the results do show that staphylococcal osteomyelitis has been produced in a large number of experimental animals. 31 rabbits in all have been used; 9 of these animals died shortly after they had been infected with living staphylococci, before sufficient time had elapsed to allow the development of bone lesions recognizable with the naked eye. Of the 22 animals in which the infection assumed a subacute course, lasting from 1 to 3 weeks, 18, or 81.8 per cent showed definite macroscopic evidence of bone inflammation. In the cases where frank suppuration and advanced bone destruction were absent, microscopic examination of stained smears taken from the bone marrow, in every case supported the slight macroscopic evidence such as subperiosteal hyperemia and visible sponginess of

the bone. The bone lesions varied from mere hyperemia and softening of the bone, with multiple subperiosteal bleeding points, to advanced abscess formation with suppuration and extensive bone destruction.

Fifty-five bone lesions were present in the 18 animals showing evidence of bone inflammation; of these 47 occurred near the ends of the long bones, yet only 8 of these were associated with a purulent arthritis; moreover, from an examination of the sites of bone destruction in the isolated bones, and from the appearance of the articular cartilages, we feel that the purulent arthritis, when present, was probably secondary to the bone lesion, being caused by a spread of the inflammatory process in the metaphysis through the epiphysis and articular surface. A serous effusion into neighboring joint cavities was, however, a fairly common concomitant of metaphyseal lesions, while in one case (rabbit 19) a purulent arthritis was present without any coexisting bone inflammation.

Several points of interest have emerged suggesting a close similarity between the experimental condition and the disease as occurring in children. Firstly, the characteristic predilection of the organism to produce inflammation in the metaphyses of the long bones, particularly at the upper end of the tibiofibula and at the lower end of the femur. Secondly, the relative infrequency of spread of a metaphyseal abscess through the epiphysis into the neighboring joint cavity. Thirdly, the pyrexial course of the infection in the acute stage, with lapse into a chronic condition of advanced suppuration in those animals that survived the acute stage. And, fourthly, in chronic cases the presence of multiple bone abscesses.

Multiple abscesses in the kidneys, often bilateral, were present in the majority of cases, but the muscles and other viscera were usually strikingly free from signs of inflammation.

In view of the ease with which the results described in the present paper were obtained, it is surprising that the literature on the subject should contain so many conflicting results, but from the present work it seems possible that at least three factors are of importance in the experimental production of staphylococcal osteomyelitis. Firstly, it would appear that the particular strain of staphylococcus used may play a deciding rôle in the production of the condition experimentally;

whether or not the strain of staphylococcus used in this study possesses a special affinity for bone tissue, in other words, whether there exist "osteo-strains" of this bacterial species, must remain, for the present, an open question. Secondly, it appears to be of importance in facilitating the detection of early lesions to arrange the experimental conditions in such a way as to bring about a relatively prolonged type of infection, and the results reported here suggest that preliminary immunization may be a decided help in achieving this end by rendering the animals more resistant to the early lethal effects of large doses. And thirdly, there are indications suggesting that the use of young rabbits, with a high degree of vascularity in the growing metaphyses, influences favorably the production of the condition, a fact that is borne out when the age incidence of staphylococcal osteomyelitis in human beings is recalled.

SUMMARY

1. The conditions under which a certain strain of staphylococcus (OH 172) causes in rabbits the development of bone inflammation have been described.

2. The virulence of the strain for rabbits was markedly raised by passage through this animal species, and especially after the culture had been recovered from a bone abscess.

3. The results indicate that it is possible to produce consistently inflammation of the bones of rabbits by the mere intravenous injection of a suitable strain of staphylococcus, without resorting to any elaborate operative technique designed to localize the organisms in the bones. It appears also that the inflammatory process so produced bears a close resemblance to staphylococcal osteomyelitis as occurring in human beings.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Right knee of rabbit 9, 24 days after infection, no abnormality.

FIG. 2. Left knee of rabbit 9, 24 days after infection; area of rarefaction present at upper end of tibia.

FIG. 3. Left tibiofibula of rabbit 9 showing area of rarefaction at upper end of tibial portion.

FIG. 4. Right knee of rabbit 13, 24 days after infection; thickening of fibula showing rounded area of rarefaction.

FIG. 5. Left knee of rabbit 13, 24 days after infection; localized area of rarefaction at upper end of tibia.

FIG. 6. Right tibiofibula of rabbit 13; thickening and rarefaction of fibular portion.

FIG. 7. Left tibiofibula of rabbit 13; rarefaction of compact bone at upper end of tibial portion.
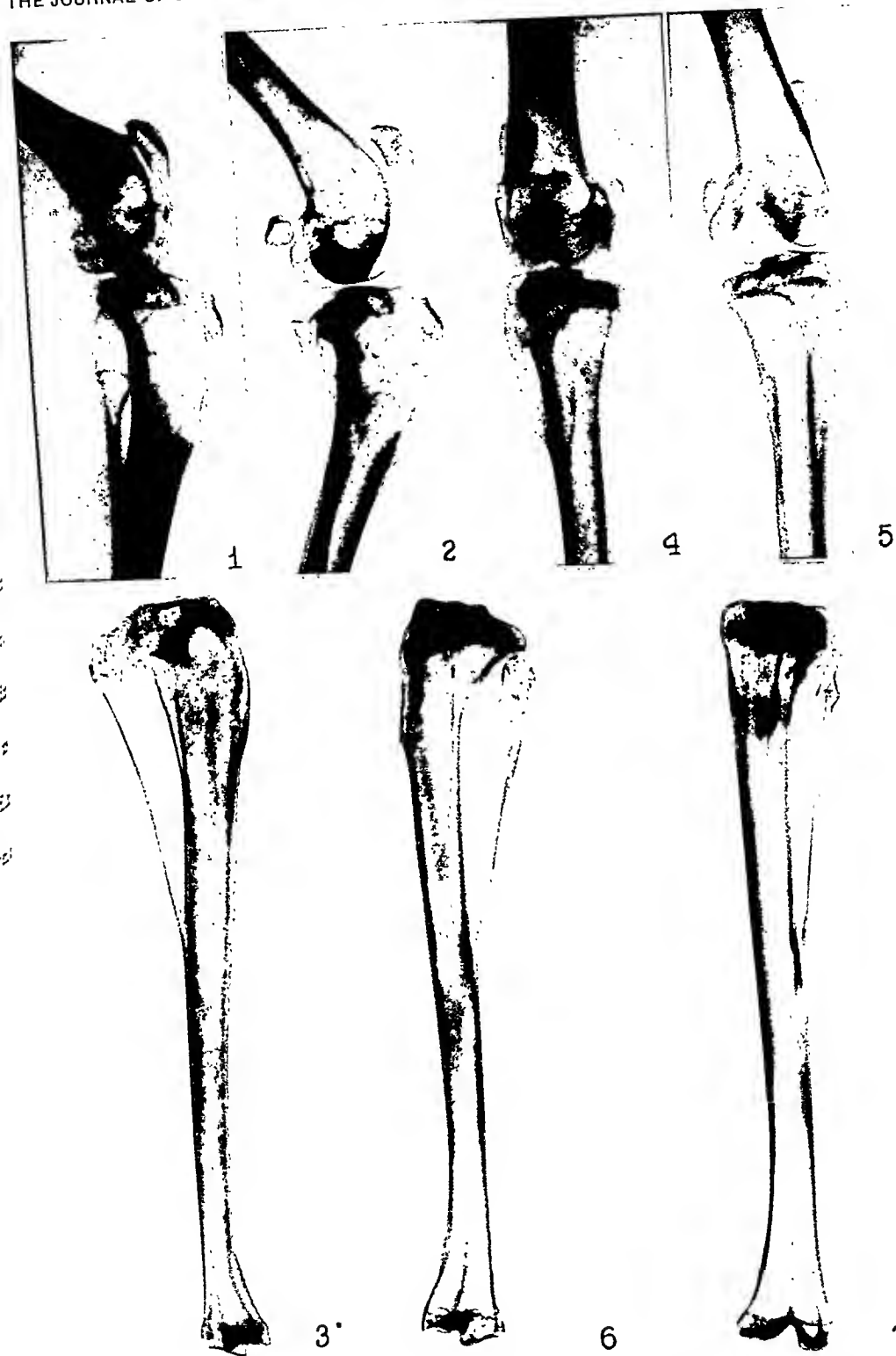
(Thompson and Dubos: Experimental staphylococcus osteomyelitis)

PLATE 5

FIG. 8. Right knee of rabbit 24, 7 days after infection; rarefaction at lower end of femur and at upper end of tibia.

FIG. 9. Left knee of rabbit 24, 7 days after infection; no abnormality.

FIG. 10. Right knee of rabbit 24, 13 days after infection; changes more advanced than in Fig. 8; suggestion of sequestration in femoral lesion.

FIG. 11. Left knee of rabbit 24, 13 days after infection; rarefaction at medial aspect of lower end of femur; also erosion of upper end of fibula.

FIG. 12. Right femur of rabbit 24; lesion with appearance of sequestration at lower end.

FIG. 13. Right tibiofibula of rabbit 24; rarefaction at upper end of tibial portion.

FIG. 14. Left tibiofibula of rabbit 24; irregularity at upper end of fibula; rarefaction at medial aspect of lower end of tibiofibula.

FIG. 15. Left femur of rabbit 23; rarefaction at lower end above the medial condyle; sequestrum visible.

SENSORY NEURON DEGENERATION IN VITAMIN DEFICIENCY

DEGENERATION OF THE POSTERIOR COLUMNS OF THE SPINAL CORD, PERIPHERAL NERVES, AND DORSAL ROOT GANGLION CELLS IN YOUNG PIGS FED A DIET CONTAINING THIAMIN (B₁) AND RIBOFLAVIN BUT OTHERWISE DEFICIENT IN VITAMIN B COMPLEX*

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Since Eijkman demonstrated, in 1897, that atrophic degeneration of the medullary sheaths of nerves occurs in hens fed a diet of polished rice, attempts have been made to produce lesions of the nervous system experimentally by deficient diets. The earliest studies were concerned largely with deficiency of the antineuritic vitamin, B₁, but in recent years vitamin A and the B₂ complex have received attention. These investigations were in the main handicapped by inadequate information regarding the vitamins and by the error of attempting to produce absolute deficiency of the factor studied rather than a partial one. Such animals often died before clear cut lesions developed. Furthermore, drawings and retouched photographs were offered as evidence of nerve and spinal cord lesions, and it was later admitted that at least some of the supposed degenerations were artefacts. So unconvincing was this earlier work, that Grinker and Kandel (1) in 1933, after negative experiments of their own, decided that long standing severe vitamin A, B₁, and B complex deficiency causes no well defined histologic changes in the central nervous system.

In spite of this criticism, the subject has been pursued by several workers, particularly by Zimmerman and his associates (2–5). They have offered further evidence that changes in the medullary sheaths of peripheral nerves and of scat-

tered fibre tracts in the spinal cord, follow chronic deficiency of vitamin A (2, 3) and that similar changes, together with degeneration of axis cylinders, follow deficiency of the heat-stable vitamin, B_2 (4). In a recent report (5) which appeared while the present work was in progress, Zimmerman and his coworkers described changes in the peripheral nerves, the posterior columns of the spinal cord, and the posterior nerve roots of adult dogs. Their experiments were well controlled and the animals carefully studied. The changes in the spinal cord, however, were not very marked. No description is given of the dorsal root ganglia.

The following observations were made in the course of an attempt to produce in young animals a condition similar to pernicious anemia. Such animals were chosen because it had been found that the blood of the mammalian fetus as well as the bone marrow, is similar to that of patients with this disease and during development undergoes changes similar to those caused by liver extract in pernicious anemia (6). The pig was used, as it is from the liver and the stomach of this animal that substances for the treatment of pernicious anemia are derived. Furthermore, it had been found that the content of anti-anemic principle in the liver of pig fetuses is very low as compared with that of adult animals (7). Consequently it was assumed that a condition resembling pernicious anemia might be produced more readily in the young pig than in other animals.

Methods

The basal diet (Table I), the ratio of protein to carbohydrate and fat, the amount of food and the salt mixture given, were determined after many trials in young animals, details of which are given elsewhere (8). Until the animals were 4½ months of age, acid-washed casein was used but after this time the crude casein was given, as it was found that the crude casein failed to support growth in rats when given without yeast. Butter was the chief source of fat at first but, at the times indicated in the charts, it was later replaced by lard. The amount of food shown in Table I represents a kilo unit, this amount, or a multiple of it, being given daily per kilo of body weight. The food was mixed with a small quantity of water and offered as a gruel.

Vitamins A and D were furnished in fresh cod liver oil (Mead Johnson, 1800 units A, 175 units D per gram) and for a short time, as above indicated, A was available in butter as well. Vitamin C was given in the quantities shown in the charts by administering orally a freshly made solution of cevitamic acid (Squibb)[1] by means of a medicine dropper three times a week. Vitamin B was furnished

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[1] Furnished through the courtesy of Dr. George A. Harrop.

by giving whole dehydrated yeast (Northwestern) in the amounts shown in the charts.

All the animals which are the subject of this report were Duroc Jersey pigs of the same litter.[2]   One (A 4-60) was received at 10 days of age, whereas the remainder were 23 days old on arrival in Baltimore.

That the diet was adequate as long as sufficient yeast was given, is indicated by comparison with the growth curve of the control animal (A 4-67) which received the same number of calories in cow's milk and sugar, supplemented with cod liver oil and cevitamic acid.   The growth curves for these animals during the period when sufficient yeast was furnished, also compare well with the growth of pigs on optimal diets in an agricultural experiment station (9).

As shown in the charts, once growth and development had been well established, the quantity of yeast was gradually decreased.   From this time, thiamin (vitamin B_1, betaxin)[3] and, in one animal, riboflavin (lactoflavin)[3] were adminis-

<div align="center">

TABLE I

Basal Diet.   One Kilo Unit

</div>

	gm.
Casein (new process, Sheffield) (protein 84.4 per cent)	9.5
Sucrose	21.0
Butter or lard (washed)	4.0
Cod liver oil	0.5
Salt mixture	2.2
Total	37.2

Caloric value 156.5.

tered by intramuscular injection twice a week in the amounts shown in the charts. These amounts were thought to be adequate or in excess of the requirement for these substances (10, 11).

Studies of the blood were made each week or oftener, and the ascorbic acid content of the blood[4] was determined at frequent intervals.   Gastric analyses were carried out from time to time.   These will be discussed elsewhere (8).   The animals were observed daily and, when neurologic signs developed, photographs and cinematographic records as well as neurologic examinations were made.

Pathological Studies.—Complete autopsies were performed.   The tissues of the nervous system were fixed immediately after removal in 10 per cent neutral formol-saline.   Whole blocks were taken of the brain through the level of the

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[2] Furnished by the Bureau of Animal Industry, United States Department of Agriculture, Beltsville, Maryland.

[3] Furnished by the Winthrop Chemical Company.

[4] Carried out by Dr. Laslo Kajdi, Department of Pediatrics.

mamillary bodies and including the basal ganglia, the pons, upper and lower medulla including the cerebellum, and at various representative levels of the spinal cord. The spinal roots and dorsal root ganglia were also removed from various levels and pieces of the brachial, sciatic, peroneal, and tibial nerves were obtained. All blocks were embedded in paraffin and stained with hematoxylin and eosin, thionin (for cells), and the Mahon modification (12) of the Weigert stain (for myelin). The Mahon technique permits uniform treatment of tissue from experimental animals and controls, and eliminates differentiation of individual sections. Glia were stained by Mallory's phosphotungstic and hematoxylin method and axis cylinders by the Bielschowsky technique. Frozen sections of the spinal cords and peripheral nerves were stained for fat with Sudan III.

RESULTS

Protocols, growth curves, and blood findings in four animals are shown in Charts 1 to 4. Except when otherwise indicated on the charts, the animals ate well and consumed all their food in a short time. Only in the last month was their appetite seriously affected, but even then the day's food was usually entirely consumed in the course of 24 hours. In pig A 4-60 (Chart 1), at 70 days of age the hind legs became weak and by the 73rd day the animal was unable to stand; on this day thiamin, 25 mg., was injected. The next day the animal was able to walk and within a week had completely recovered. Following this, the history of the three animals receiving the artificial diet was the same. As the quantity of yeast given was gradually reduced, weight gain diminished, ceased, or loss occurred. Their hair became dirty and matted. The administration of thiamin (vitamin B$_1$) was followed by a resumption of growth. This effect was temporary, however, even in spite of the excessive amounts which A 4-60 received during its last month. Pig A 4-64 (Chart 2) received riboflavin continuously during the latter half of its life period. This too failed to delay the onset of the signs to be described, or to prevent death.

First in pig A 4-60, then in A 4-64 and A 4-65, at the time indicated on the charts (N.S.), a picture developed which was similar in each (Figs. 1, 2, 3). Noticed casually as an awkwardness in the hind legs while walking, on more careful observation it was seen that each pig had developed a peculiar slapping gait, in which the hind legs were placed widely apart rather than close together as in the normal animal. The ataxia progressed and the animals stumbled frequently. They
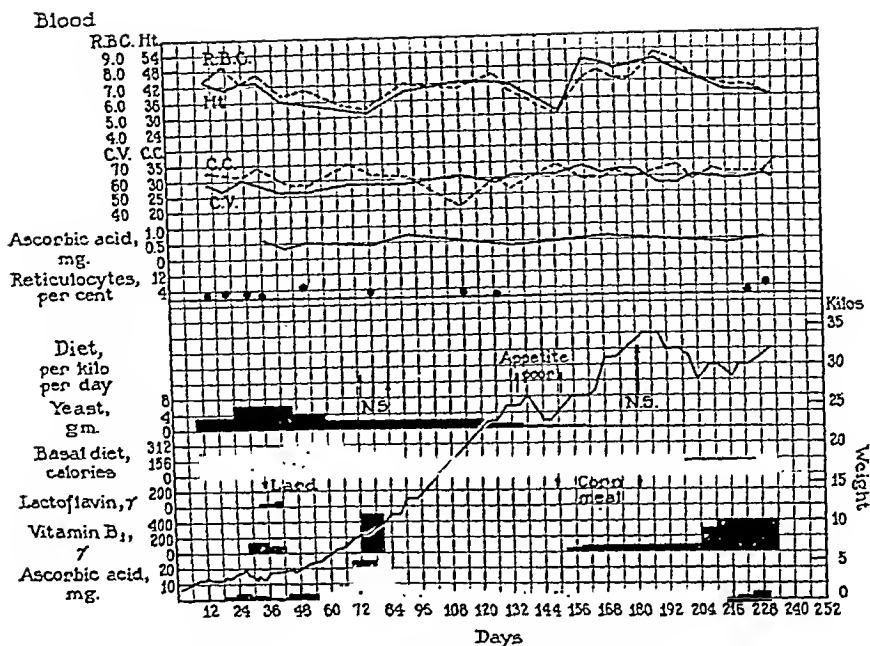
CHART 1. Diet, growth curve, and blood findings in pig A 4-60, male.

Legends.—All dietary data are shown as the amount per kilo body weight daily. At the point indicated, lard replaced butter in the basal diet. The quantities of thiamin (B_1) and riboflavin (lactoflavin) given are shown as micrograms per kilo per day, although actually they were injected intramuscularly twice each week.

The red cell counts (R.B.C.) are represented in millions per c.mm.; the volume of packed red cells (Ht.) in cc. per 100 cc. blood; the mean corpuscular volume (C.V.) in cubic microns; and the mean corpuscular hemoglobin concentration (C.C.) in per cent.

Protocol.—The quantity of yeast given was successively reduced from 6 gm. per kilo daily, to 4 gm., 2 gm., 0.5 gm., 0.2 gm., and finally 0.1 gm. A total of 373 gm. were given per kilo, an average of 1.6 gm. per day. The total amount of thiamin given was 540 mg., of which 360 mg. was given during the last month. Only 22.5 mg. of riboflavin were given. During the period between arrows marked corn meal, this animal received 15 gm. yellow corn meal instead of the same weight of sucrose in its basal diet.

A brief and temporary early period of weakness (see text) is represented between two arrows. The onset of the marked ataxia described in the text is indicated by a long arrow near the right (N.S.)

Autopsy.—324th day. Animal killed by pentobarbital. Body moderately well nourished, hair a little dry but only slightly matted. Nervous system as described in the text (Figs. 5, 7, 8, 10, 16, 17, 18). Thoracic and abdominal organs normal, except for numerous pericardial adhesions, presumably caused by repeated cardiac punctures.

211

were particularly awkward in turning. One of the most striking features was a clonic scratch-like movement of one or other hind leg which often persisted for several seconds while the pig stood on its other feet. As time went on the animals became more and more reluctant to move about, and sat with their hind legs spread widely
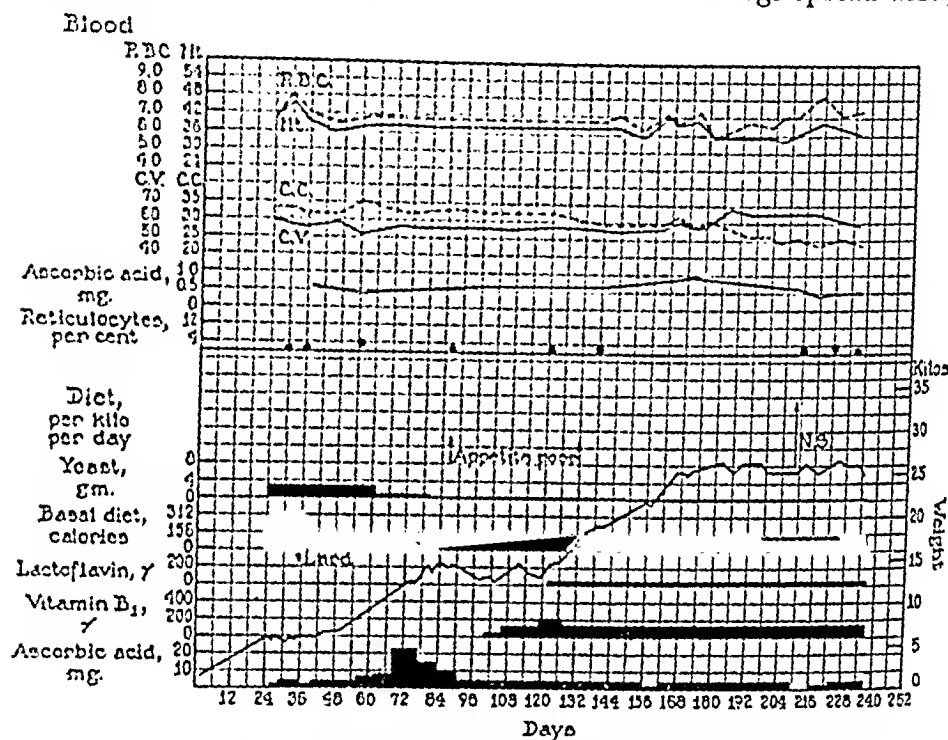
CHART 2. Diet, growth curve, and blood findings in pig A 4-64, female.

Legends.—Same as in Chart 1. N.S. denotes the onset of marked ataxia.

Protocol.—The quantity of yeast was successively reduced from 3 gm. per kilo daily, to 1 gm., 0.5 gm., 0.2 gm., and finally 0.1 gm. A total of 169 gm. were given per kilo, an average of 0.7 gm. per day. The total amount of thiamin given was 415 mg. and the total quantity of riboflavin was 103.8 mg.

Autopsy.—238th day. Animal found dead. Nervous system described in the text (Figs. 3, 11, 14). The thoracic and abdominal organs showed no changes. No cause of death was found.

apart or even sprawled forward beyond their fore legs. The effect of blindfolding on the gait and balance was tested, in order to see if the loss of visual control caused any greater defect. At the time this was done, however, the animals were already so ataxic that little difference was noticed.
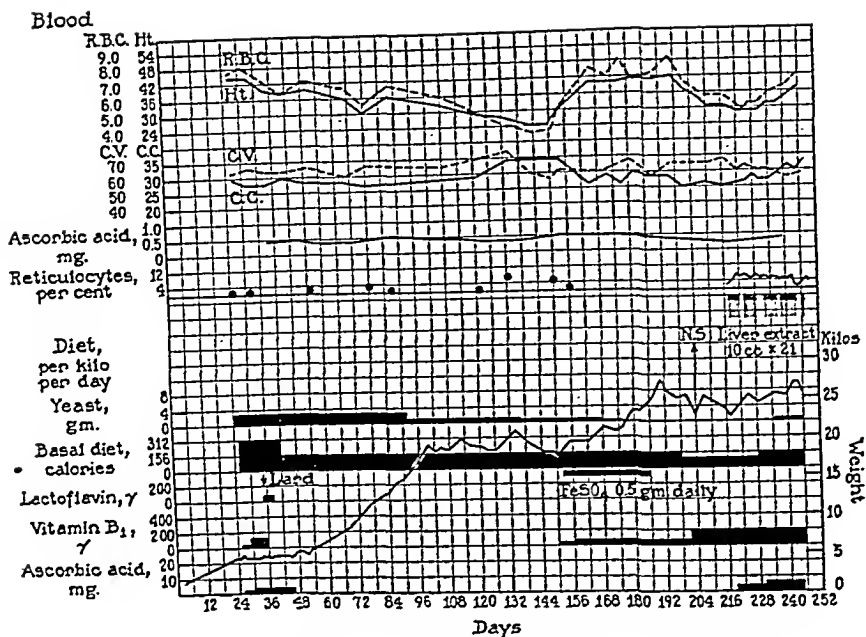
CHART 3. Diet, growth curve, and blood findings in pig A 4-65, female.

Legends.—Same as in Chart 1.

Protocol.—The quantity of yeast was successively reduced from 3 gm. per kilo daily, to 0.5 gm., 0.3 gm., 0.2 gm., and finally 0.1 gm. During the last 12 days, after 13 injections of liver extract had been given, the quantity of yeast was increased to 0.3 gm. A total of 247 gm. were given per kilo, an average of 1 gm. per day. The total amount of thiamin given was 312 mg., and the total quantity of riboflavin was 3 mg.

Autopsy.—247th day. Animal found dead. Ulcers between hind hoofs. Round firm fluctuant mass over the left buttock containing thick yellow pus. Tissues of the nervous system described in the text (Figs. 12 and 15). Muscles edematous and in places hemorrhagic in appearance. Abdomen distended by gas in the stomach and bowel; no gas or fluid found in the peritoneal cavity. Liver small and pale; spleen normal. Cut surface of the kidney showed mottling in the cortex. Right pleural cavity largely obliterated, but loculated areas contained pus; left cavity normal. Both lungs rather nodular, the right much more so than the left. General pulmonary congestion, no consolidation. Considerable enlargement of the root glands on both sides. Heart normal except for pericardial adhesions.

Histological studies showed abscesses in the lungs and kidneys. None found in the liver or spleen, nor in any of the nervous tissues examined. The small vessels in the affected organs contained enormous numbers of a large Gram-positive anthrax-like bacillus. Unfortunately no material was taken for cultural studies so that identification of the organism was not possible. The septicemia was thought to be entirely a terminal event and unassociated with the nervous condition. It is noteworthy that this animal was given very little ascorbic acid.

213

In spite of their locomotor disabilities the motor power was not greatly diminished, as judged from the animals' movements when venepunctures or gastric analyses were performed. There was no sign of muscular wasting. The tendon reflexes were absent or only doubtfully obtained. From the ataxia, loss of reflexes, and intact motor power, it was evident that a sensory defect existed. Further-
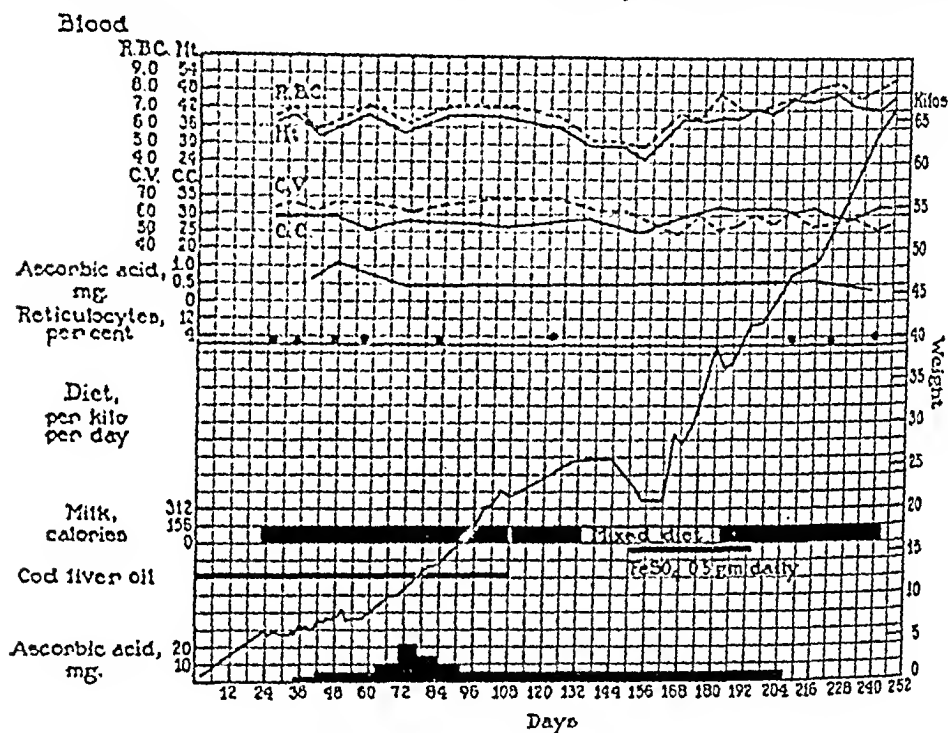
CHART 4. Diet, growth curve, and blood findings in pig A 4-67, female.

Legends.—Same as in Chart 1.

Protocol.—For 108 days this animal received cow's milk and sugar, 156 calories per kilo daily. After this time it received a mixed diet consisting of waste from the hospital kitchen. The cod liver oil was given in amounts of 0.5 gm. per kilo daily.

Autopsy.—247th day. Tissues of the nervous system as described in the text (Figs. 6, 9, 13, 19). The thoracic and abdominal organs were normal, both in the gross and microscopically.

more, ulcers developed on the hind limbs and above and between the hoofs, and these seemed to be insensitive to pin pricks. On pinching of the tendons, the animals showed no discomfort.

In marked contrast was the gait and general appearance of the control animal (A 4-67) (Fig. 4). Not only was this pig larger and heavier

but the body and hair were clean, and no ulceration occurred.   Tendon reflexes were readily elicited.   The animal moved about quickly and naturally with the hind feet close together.   Blindfolding had no effect on the animal's movements and it could swing about with ease.

In none of the animals were cranial nerve changes observed: vision, eye movements, swallowing, and voice were normal.   The jaws were strong and their snouts were sensitive to pin pricks.

Pigs A 4-60 and A 4-67 were killed by the intravenous injection of pentobarbital but A 4-64 was found dead.   It was decided to attempt treatment of A 4-65 by the intramuscular injection of a crude liver extract (campolon, Winthrop Chemical Company) (Chart 3).   Slight improvement followed.   The animal finally died, however, of septicemia.

Pathological Studies.—The findings of main interest were in the nervous system.

1. Peripheral Nerves.—In general the changes found were the same in the three affected animals (pigs A 4-60, A 4-64, A 4-65), although they differed in severity.   Demyelination was extreme in the sciatic nerves and their branches (Figs. 14 and 15).   In the brachial nerves this was also present but was less extensive (Fig. 17).   In pig A 4-60, which developed neurological signs before the others, the demyelination was most advanced.   It was observed in the transverse sections that certain bundles showed almost complete loss of myelin whereas others were little changed (Fig. 17).   The significance of this will be discussed later.   With the loss of myelin a considerable increase in the number of neurilemma cells was seen and they were frequently found enlarged and swollen.   This was most marked in A 4-65, the animal which died of terminal septicemia.   Everywhere the loss of myelin was more widespread than the damage to the axis cylinders.   The latter were swollen and in many places fragmented (Fig. 18); the changes again were more marked in A 4-60 than in A 4-64 or A 4-65.

Pig A 4-67 (control) showed an entirely different picture (Figs. 13 and 19).   Myelin stained consistently in all the nerves examined, appearing in the transverse sections as regular rings (Fig. 13).   The neurofibrils appeared as continuous, heavily impregnated fibres and the neurilemma cells were seen as very thin fusiform nuclei lying parallel to and along the myelin sheaths (Fig. 19).

2. Spinal Roots and Posterior Root Ganglia.—Here again the normal appearance of cells and fibres in pig A 4-67 contrasted with the varying degrees of demyelination and chromatolysis in the three animals A 4-60, A 4-64, and A 4-65.   As before, the changes were most marked in pig A 4-60.   The extreme loss of myelin in the posterior root fibres contrasted strikingly with the normal anterior roots

(Figs. 7 and 8). This was seen in all the spinal roots examined including those from the cervical region as well as those in the sacral, although it was less marked in the former. This finding perhaps explains the fact referred to above, namely that in the peripheral nerves some bundles appeared well myelinated while others showed almost complete degeneration. In other words the demyelination appears to have been confined to the afferent fibres. The ganglion cells in the three animals which were given a deficient diet showed changes which varied in degree from some loss of Nissl substance, to severe chromatolysis. The cells of the control animal showed a large central nucleus, with a deeply stained nucleolus, and rather heavy Nissl granules (Fig. 6). The ganglion cells in the affected animals appeared to be diminished in number and many showed little or no Nissl substance except around the nucleus (Fig. 5). In some cells, the nuclei were eccentric and no Nissl substance whatever was observed. In pig A 4-60 alone, however, was chromatolysis frequent and well developed.

3. Spinal Cord.—The uncut cord appeared normal to the naked eye. On looking at the cut transverse surface, the posterior columns stood out clearly by their white color which contrasted with the more yellow appearance of the rest of the cord. The sections of the spinal cords in pigs A 4-60, A 4-64, and A 4-65, in the myelin stain showed a definite pallor of the posterior columns (Figs. 10, 11, 12). Throughout the posterior columns large round, punched-out areas were seen and in these spaces no axis cylinders were apparent. There was considerable variation in the size and shape of the myelin rings. This was in marked contrast to the regularity of the myelin sheaths and the central position of the axis cylinders in the lateral and anterior columns of these three animals, and in all parts of the cord in A 4-67 (Fig. 9). Stained by Sudan III, some globules of free fat were seen in the posterior columns. Although with the Mallory stain some glial growth was observed in the posterior columns, nowhere was it conspicuous and there was no dense scarring. Examination of the cells in the anterior horns revealed no abnormalities. At most there was some vacuolation of these cells in the affected animals which was not present in the control pig. The cells of the dorsal nuclei and substantia gelatinosa Rolandi showed no changes. The membranes and blood vessels of the cord were normal except for the injection of the latter in the animal which died of septicemia (A 4-65).

4. Brain.—With the exception of the changes previously described in the posterior columns, which could be followed as high as the cuneate and gracile nuclei in the lower medulla, the brain stem and brain were quite normal.

In summary, the process seemed to consist of a quite selective degeneration of the peripheral sensory neuron involving its cell body in the posterior root ganglion, the peripheral axon, and the central axon included in the posterior roots and in the posterior columns. No clear cut abnormalities were seen in the anterior roots or anterior horn cells and many bundles of well myelinated fibres, probably motor, were evident in sections of the peripheral nerves.

The tissues other than those of the nervous system were either normal, or showed the results of secondary infection. Details are given in the descriptions of Charts 1 to 4.

DISCUSSION

The neurological signs in these animals were so well marked, the changes in the nervous system so well defined, and there was such a close correlation between the signs and the histologic findings that there can be no doubt regarding the pathologic lesion. Selective degeneration of the sensory neuron occurred.

It is thought that the changes in the nervous system were dependent upon a dietary deficiency of one or more components of the vitamin B complex, other than thiamin (B_1) or riboflavin. The animals received a diet which was adequate in proteins, carbohydrates, fats, unsaturated fatty acids, minerals, and vitamins A, C, and D, and which was supplemented at first by adequate amounts of yeast. As the quantity of yeast was gradually reduced, growth ceased. The appetite of the animals became poor, although they still consumed their daily ration during the 24 hour interval between feedings. No neurological signs were observed, however. Shortly after administration of thiamin was started, appetite and growth were restored. It was not until several months had elapsed that the described neurological signs appeared. In one of these animals thiamin was given even in excessive amounts. We believe, therefore, that the disorder could hardly have been the result of insufficient vitamin B_1.

The neurological changes did not seem to be caused by a lack of riboflavin, for A 4-64 developed well marked neurological signs similar to those observed in pigs A 4-60 and A 4-65. It may be mentioned, however, that in this animal the neurological signs and the changes found in the tissues of the nervous system, were less marked than in the animals which received very little riboflavin, in spite of the fact that it had received less yeast than A 4-65. In experiments which are now in progress, the significance of riboflavin, nicotinic acid, vitamin B_6 and other components of the B_2 complex, is being studied, and the possibility of preventing the development of the described neurologic changes by the administration of large amounts of yeast, is being investigated.

The relation if any, of the neurological changes observed to those

occurring in nutritional disorders in man, such as pernicious anemia, and pellagra, is at present a matter only for speculation. It is of interest, however, that the diet which these animals received seems to be low in its content of "extrinsic factor" (8). Comparing the clinical picture in the affected animals to the common neurologic disorders of man, the similarity with that of tabes is striking. In view of the ataxia, the animals immediately impressed one as having predominant posterior column pathology rather than a peripheral neuritis or subacute combined degeneration. There was no evidence of spasticity.

Pathologically the picture resembles none of the diseases mentioned above. The degeneration of the central axon and cell body is not a part of the usually recognized pathology of sensory neuritis in man. In tabes dorsalis, degeneration of the posterior root fibres and posterior columns, as well as replacement by a dense glial scar is seen, but lesions have not been reported in the peripheral axon or posterior root ganglion. In the animals here described, the whole neuron was involved and glial reaction was minimal. The peripheral neuritis and posterior column changes without glial reaction are quite similar to those seen in subacute combined degeneration of the cord, but in no instance were any changes observed in the lateral columns. In human beings, the lateral columns as well as areas in the anterior columns, and even in the brain stem and brain, may be involved. These animals also failed to show the diffuse degeneration of the cord, the pigmentary changes in the ganglion cells, or the π granules of Wilson in the peripheral nerves, which have been described in pellagra.

It must be kept in mind, when comparison is made between changes produced in animals by experiment, and those lesions discovered in man, that in human beings nutritional deficiencies are usually multiple in character. Dietary defects exactly comparable to that produced in the animals here described, may not occur.

SUMMARY

Young pigs were given an artificial diet presumably adequate in all respects. As they developed, the quantity of yeast was gradually reduced while thiamin (vitamin B₁) and riboflavin were given in its place. The rate of growth decreased, the general condition of the

animals became impaired, and marked ataxia without motor weakness developed. Histologically, severe degeneration of the posterior columns of the spinal cord, the dorsal root ganglion cells, and the peripheral nerves was found.

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EXPLANATION OF PLATES

PLATE 6

FIGS. 1 to 4. Reproduced from cinematograph film; showing wide base of support used by affected animals (A 4-65, Figs. 1 and 2, A 4-64 Fig. 3); the relative size and stance of the control animal (A 4-67) are shown in Fig. 4.

FIG. 5. Pig A 4-60. Posterior root ganglion cells showing eccentricity of nucleus and loss of granules except around the nucleus. Nissl stain, thionin. × 300.

FIG. 6. Pig A 4-67 (control). Posterior root ganglion cells as in Fig. 5, showing central nucleus and normal Nissl substance.

FIG. 7. Pig A 4-60. Anterior and posterior roots, posterior root ganglion, longitudinal section. Myelin normal in anterior roots; largely lost in posterior. Mahon stain. × 100.

FIG. 8. Pig A 4-60. Anterior and posterior roots, transverse section. Anterior root shows normal myelin; the posterior root is somewhat demyelinized.

PLATE 6

FIG. 9. Pig A 4-67 (control). From left to right, transverse sections of spinal cord of the upper thoracic, lower cervical, sacral, and lumbosacral regions. Mahon stain. × 5.

FIG. 10. Pig A 4-60. Spinal cord (same order as in Fig. 9): pallor of posterior columns at all levels, indicating loss of myelin. Mahon stain. × 5.

PLATE 7

9

10

(Wintrobe *et al.*: Neuron degeneration in vitamin deficiency)
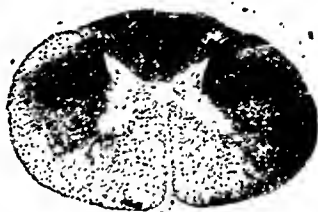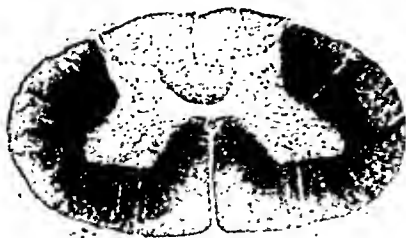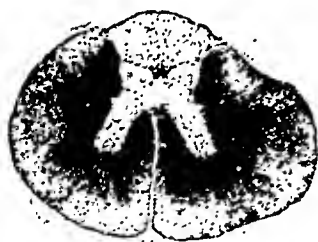
FIG. 11. Pig A 4-64. Spinal cord (same order as in Fig. 9): pallor of posterior columns. Mahon stain. × 5.

FIG. 12. Pig A 4-65. Spinal cord (same order as in Fig. 11): pallor of posterior columns. Mahon stain. × 5.

In all these spinal cord sections the slight peripheral pallor is considered an artefact.
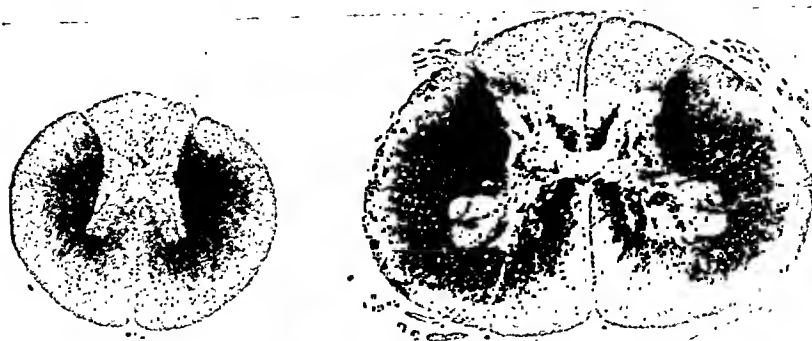
(Wintrobe *et al.*: Neuron degeneration in vitamin deficiency)

PLATE 9

FIG. 13. Pig A 4-67 (control). Sciatic nerve: normal myelin in all nerve bundles. Mahon stain. × 100.

FIG. 14. Pig A 4-64. Sciatic nerve: almost no myelin in nerve bundles. Mahon stain. × 100.

FIG. 15. Pig A 4-65. Sciatic nerve: again severe demyelinization (the black spots are dilated capillaries with red cells stained black). Mahon stain. × 100.

FIG. 16. Pig A 4-60. Peroneal nerve: extreme loss of myelin. Mahon stain. × 100.

FIG. 17. Pig A 4-60. Brachial nerve: compare with Fig. 16. Demyelinization is not so severe. Mahon stain. × 100.

FIG. 18. Pig A 4-60. Sciatic nerve: swelling and fragmentation of axis cylinders. Bielschowsky stain. × 300.

FIG. 19. Pig A 4-67 (control). Sciatic nerve: axis cylinders uniform and continuous. Bielschowsky stain. × 300.

PLATE 9
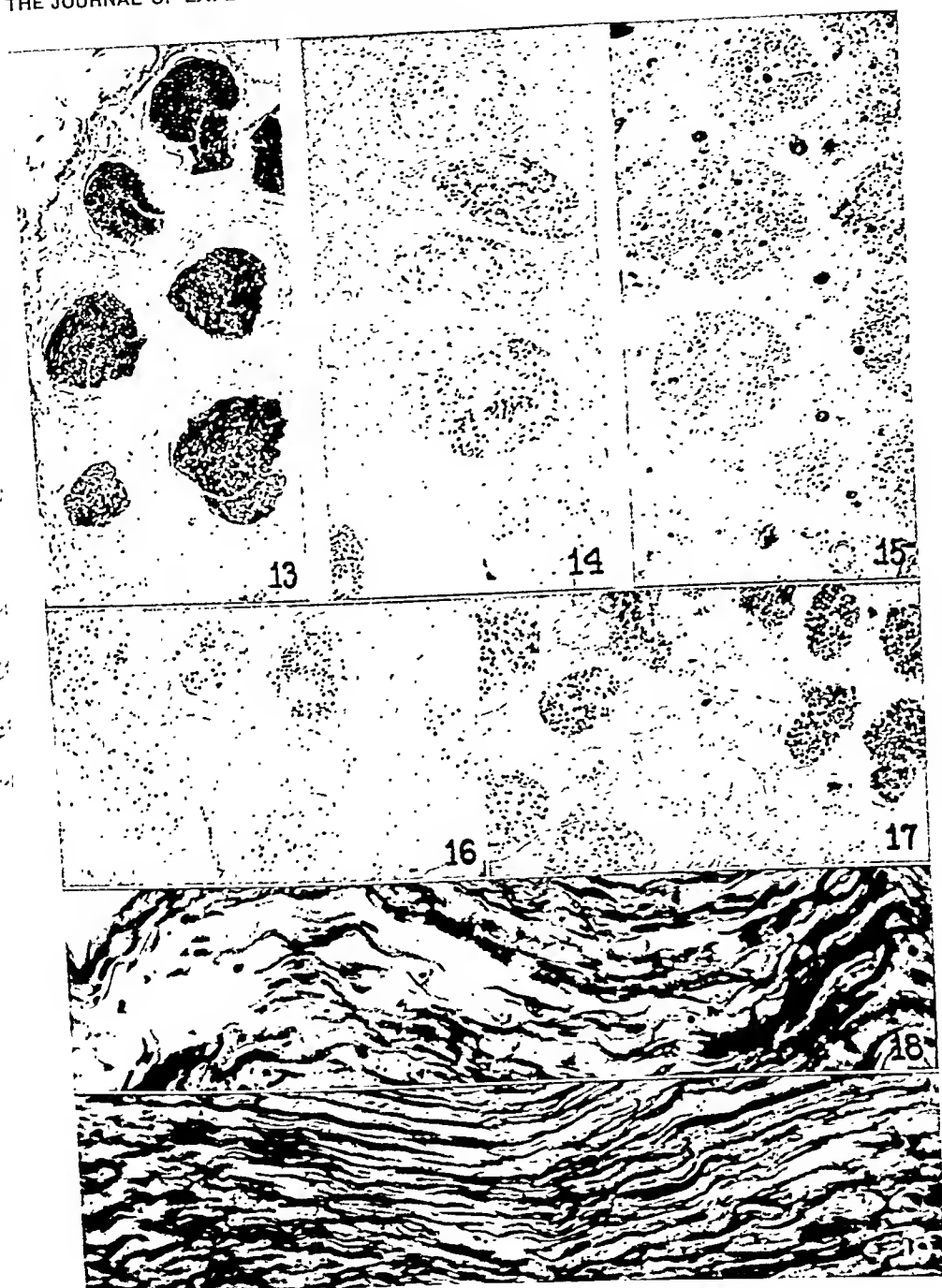
THE ISOLATION OF THE BLOOD GROUP A SPECIFIC SUB-STANCE FROM COMMERCIAL PEPTONE

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As part of an investigation on the chemical constitution of the capsular polysaccharide of Type III Pneumococcus we have been engaged during the past year in the preparation of large quantities of the specific carbohydrate. This substance has been isolated from autolysates of Type III pneumococci grown in meat infusion broth containing 1 per cent commercial peptone. The method of purification was that described by Heidelberger, Kendall, and Scherp (1). When precipitation of the specific carbohydrate as the copper salt was omitted, it was found that the Type III polysaccharide isolated was contaminated in the gross, in some instances with as much as 60 per cent of a substance having many characteristics of a polysaccharide, serologically inert in Type III antipneumococcus horse serum. It was considered of interest, therefore, to separate and identify this substance and to trace its origin.

The polysaccharide inert in antipneumococcus serum can be separated from the type specific pneumococcus carbohydrate either by precipitating the latter as the insoluble copper or barium salt, or by direct precipitation of the specific substance in the cold at pH 1.5 (2). After removal of the specific bacterial carbohydrate considerable quantities of a polysaccharide rich in glucosamine and having a low specific rotation could be separated by alcoholic precipitation from the supernatant liquid. The similarity in chemical properties of this substance which appears to be polysaccharide in nature, to the blood group A specific substance described by Landsteiner (3) seemed so striking as to suggest that it might be identical with the latter. Serological tests and chemical analysis proved this to be the case. Since it is unlikely that the Type III Pneumococcus itself produced

this substance in such large quantity, we presumed that it came from the culture medium in which the organisms were grown. On concentrating some 50 liters of sterile broth *in vacuo* and working up the concentrate according to the method outlined (1), 3 gm. of the blood group A specific substance were obtained. At this juncture it was thought that the true source of the latter was not the meat infusion but the commercial peptone employed in preparing the medium. This proved to be the case since a substance having the same chemical properties was readily isolated from commercial peptone as described below.

EXPERIMENTAL

Preparation of the Blood Group A Specific Substance from Commercial Peptone.—
500 gm. of commercial peptone[1] and 200 gm. of sodium acetate were dissolved in 1500 cc. of warm water and a crude polysaccharide-like material precipitated by the addition of 3750 cc. of 95 per cent ethyl alcohol. After standing overnight the clear supernatant liquid was decanted, the precipitate centrifuged, and then dissolved as completely as possible in 300 cc. of water. Insoluble material was removed by centrifugation and discarded. The substance was again precipitated from the supernatant liquid by the addition of 2.5 volumes of alcohol after first adding 50 gm. of sodium acetate. The precipitate was redissolved and reprecipitated. The crude substance was once more dissolved in 200 cc. of water, 30 gm. of sodium acetate were added, and the pH of the solution adjusted to 4.8 by the addition of 25 per cent acetic acid. The solution was then deproteinized by the method of Sevag (4), using 50 cc. of chloroform and 10 cc. of butyl or amyl alcohol until a portion of the supernatant liquid no longer gave a protein test on saturation with picric acid. In the case of the neopeptone, only two treatments were necessary, whereas the Pfanstiehl peptone required five treatments. The material was finally precipitated from the deproteinized solution by the addition of 2.5 volumes of alcohol. The precipitate was dissolved in 100 cc. of water, passed through a Berkefeld V filter, and the filtrate dialyzed for several days against successive changes of distilled water. The clear colorless solution (faintly yellow in the case of Pfanstiehl peptone) was now poured, with stirring, into 10 volumes of acetone. The precipitated substance was collected on a hardened paper, washed with fresh acetone, and finally dried over sulfuric acid *in vacuo*. 500 gm. of neopeptone yielded 14 gm. of a substance having many of the properties of a polysaccharide. An equal quantity of Pfanstiehl peptone yielded only 4 gm. of crude carbohydrate.

[1] The two preparations of peptone studied were Pfanstiehl peptone, manufactured by the Pfanstiehl Chemical Company, Waukegan, Illinois, and neopeptone, prepared by the Difco Laboratories, Detroit, Michigan.

Chemical Properties of the Group A Specific Substance from Peptone.— The chemical properties of the two preparations, one from neopeptone and the other from Pfanstiehl peptone, are given in Table I, and compared with the properties of the group A specific substance isolated from commercial pepsin and from horse saliva by Landsteiner (3, 5). The material obtained by fractional precipitation with alcohol from a solution of neopeptone was isolated as a white amorphous powder, readily soluble in water to give a viscous faintly opalescent solution which foamed readily on shaking. A 2 per cent solution of the sub-

TABLE I

Analyses of Blood Group A Specific Substance Derived from Various Sources

Blood group A specific substance derived from	$[\alpha]_D$	Ash	C	H	N	Total S	S as SO$_4$	P	Acetyl	Reducing sugars after hydrolysis
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Horse saliva*	+10°	1.20	44.56	6.91	7.08	1.78	0.05	0.23	9.40	57.6
Fairchild pepsin†	+16°	0.0	46.88	6.62	6.16	0.08	—	0.10	9.95	7.07
Difco neopeptone	+11.5°	1.86	46.68	6.53	5.85	0.46	0.00	0.00	9.56	73.0
Pfanstiehl peptone	+9.6°	4.72	44.86	6.23	5.48	1.96	0.00	0.71	8.90	62.0

All analyses given in this table have been calculated on an ash-free basis of the various preparations.

   * Reference 5.

   † Reference 3.

stance gave a weakly positive biuret reaction, a weak xanthoproteic reaction, and a negative ninhydrin test. The solution gave a positive Hopkins-Cole test, indicating the presence of tryptophane, and a weak Sakaguchi test for arginine. The Millon test was negative as was the Tollens test for uronic acids and the Bial test for pentose. The solution gave a strongly positive Molisch test, and a strong test for glucosamine (6). The substance was not precipitated from solution by copper sulfate, lead acetate, uranium nitrate, or by barium chloride or hydroxide. Saturation with ammonium sulfate precipitated the material from solution, and high concentrations of tannic acid caused partial precipitation. In solution the substance when

strongly acidified with hydrochloric acid, was precipitated on the addition of phosphotungstic acid. Neither picric, trichloroacetic, nor sulfosalicylic acid caused precipitation. A similar solution of the blood group A substance isolated from Pfanstiehl peptone behaved as did that from neopeptone except that the former gave a positive Tollens test for uronic acid. In their qualitative reactions the two preparations appear to be very closely related. Because of the

TABLE II

Inhibition of Hemolysis of Sheep Cells in Anti-A Rabbit Serum by Blood Group A Specific Substance Derived from Commercial Peptone

Blood group A specific substance derived from	Dilution of substances tested					
	1:1,000,000	1:2,000,000	1:4,000,000	1:8,000,000	1:16,000,000	1:32,000,000
Mucin (Landsteiner)	0	0	W	S	AC	C
Difco neopeptone	0	0	0	Dis.	S	C
Pfanstiehl peptone	0	Dis.	AC	C	C	C

A control test, containing immune serum in dilution employed in above tests, complement and washed sheep cells, gave complete hemolysis.

C = complete hemolysis.
AC = almost complete hemolysis.
S = strong hemolysis.
Dis. = distinct hemolysis.
W = weak hemolysis.

fact that the two materials yield such a high percentage of reducing sugars on hydrolysis, they appear to be essentially carbohydrate in nature. The two substances have nearly the same specific rotations and their chemical analyses agree rather closely, though the Pfanstiehl peptone carbohydrate contains less carbon and more sulfur and phosphorus. Since the latter preparation gives a positive test for uronic acid, and because it has a relatively higher sulfur content, it may possibly be contaminated with chondroitin-sulfuric acid.

Serological Properties of the Blood Group A Specific Substance from Peptone.—Although the two preparations derived from peptone are

probably not pure chemical entities, they appear to be essentially free from contaminating proteins. They possess a marked inhibitory action on the hemolysis of sheep cells when added in high dilution to a system containing group A antiserum and complement, as may be seen from Table II. The titrations of activity of the two preparations were performed by Dr. Landsteiner according to the method described by him (5), and the results compared with those obtained with a partially purified preparation of the group A substance isolated by him from commercial mucin. It may be seen from Table II that the serological activity of the group A substance from neopeptone is greater than is that derived from the Pfanstiehl peptone, a fact which likewise indicates the greater purity of the former substance.

DISCUSSION

The occurrence in commercial peptone of a substance which inhibits the hemolysis of sheep cells by an antiserum to blood group A human erythrocytes has been observed by Schiff (7). Landsteiner (8) has also noted that a large number of commercial peptones are rich in this substance. The isolation and identification of this substance during the preparation of the specific polysaccharide of Type III Pneumococcus is therefore not surprising. Since the majority of peptones are prepared by the peptic hydrolysis of animal proteins, the true source of the blood group A specific substance probably resides in the commercial pepsin (3) used in the manufacturing process. Although the method of isolation of bacterial polysaccharides from cultures grown in peptone broth usually involves a selective precipitation of the specific bacterial carbohydrate with a heavy metal salt, it is not unlikely that some of our preparations have been contaminated with small amounts of the blood group A specific substance.

Witebsky, Neter, and Sobotka (9), using a preparation of Type I pneumococcus polysaccharide prepared in this laboratory, showed that this substance markedly inhibited the hemolysis of sheep cells by blood group A antiserum in the presence of complement. Samples of Types II and III pneumococcus polysaccharides, not prepared in this laboratory, likewise exhibited this property, though to a considerably less degree. The possibility that the Type I pneumococcus polysaccharide possesses this activity by virtue of a similarity in

FACTORS INFLUENCING THE PERSISTENCE OF CHORIOMENINGITIS VIRUS IN THE BLOOD OF MICE AFTER CLINICAL RECOVERY

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In previous publications (1, 2) it was reported that the virus of lymphocytic choriomeningitis persisted in the bodies of some infected mice for several weeks or months after clinical recovery, while it could not be demonstrated in others. Since the carrier problem is of considerable importance in the epidemiology of certain virus diseases and also has a bearing on the mechanism of the immunity to viruses, the factors that influence the duration of the infection in mice were investigated.

The presence or absence of demonstrable virus in the blood was chosen as a basis of comparison, since previous tests have shown that the virus can almost always be detected in the blood of recovered mice if it is present in appreciable amounts in their tissues. This is chiefly due to the absence or exceedingly low concentration of antibodies in the serum of such animals. There are exceptional cases, however, in which the blood is not infectious but virus is excreted in the urine. We have observed two such instances, mice which became infected naturally when very young and were tested for virus in the blood and urine at the age of 4 or 6 months. The kidneys tested in one animal contained virus, which probably accounted for the virulence of the urine. In spite of such exceptions it is believed that the examination of the blood for virus is adequate for comparative purposes.

Methods and Materials

Most of the technical procedures used have already been described, but some of them have since been modified and will therefore be described again.

Tests for Virus in Blood.—The blood to be tested was obtained by heart puncture under deep ether anesthesia. About 10 per cent of the mice were lost through injury, but the others did not seem to be harmed even when the bleedings were repeated at short intervals. In tests with undiluted blood from mature mice, 0.1 cc. heart blood was withdrawn with a 0.25 cc. syringe and a 27 gage needle, and 0.05 cc. was inoculated immediately into the brain of an anesthetized mouse. Mice 5 to 6 weeks of age were used as test animals. They are about equally as susceptible as guinea pigs to blood from carrier mice. The mice that failed to show symptoms after the blood inoculation were tested for immunity by intracerebral injection with highly virulent mouse passage virus given 2 weeks after inoculation, an equal number of normal mice being used as controls. A small portion of the mice so tested proved completely immune, and such animals were considered to have become infected by the blood.

For titration 0.2 cc. heart blood was withdrawn and mixed immediately with 1.8 cc. heparinized saline. Further decimal dilutions were likewise made in heparinized saline. The heparin has no inactivating effect upon the virus and is harmless to mice. The highest decimal dilution causing characteristic symptoms on intracerebral injection was considered as the titration end-point.

Tests for Virus in the Tissues, Nasal Secretions, and Urine.—The carrier mice whose viscera were tested were bled (1.2 to 1.5 cc.), the blood being collected in a test tube containing a trace of heparin. Of the organs removed immediately after death a 10 per cent suspension in saline was made, which was considered a 10^{-1} dilution. The decimal dilutions in saline indicated in Table III were kept in an ice bath during the short period of time between preparation and injection, and 0.05 cc. of each dilution was inoculated intracerebrally into a mouse. As with the blood, which was titrated simultaneously, the highest decimal dilution producing typical symptoms was considered as the titration end-point.

Nasal washings were obtained according to the method already described (1), that is, simply by immersing the nostrils of a mouse 3 times for about 20 seconds in 1 cc. saline contained in a slanted Petri dish. The animal is thus forced to inhale some fluid repeatedly and to blow it out into the remaining fluid, which gradually becomes cloudy. Severe dyspnea usually follows each of the three inhalations but the mice recover rapidly. Some care is necessary, however, to prevent the animals from drowning. The undiluted washing fluid was injected into the hind pads of guinea pigs in amounts of 0.7 cc., and the tenfold dilutions of it in amounts of 1 cc.

Urine was collected in a Petri dish by exerting slight pressure on the rear part of the abdomen in the region of the urinary bladder, and inoculated in the same way as the nasal washings. The dosage of undiluted urine varied with the amount obtained.

Experimental Mice.—The normal white mice used came from the virus-free mouse colony mentioned before (2), which was built up on the progeny of some uninfected mice obtained from the colony in which choriomeningitis was dis-

covered in 1934. This new stock has been kept under close observation, and has been free from the disease since 1935.

Further essential technical details will be given in the text.

The Influence of the Mode of Natural Infection on the Persistence of Virus in the Blood

Choriomeningitis in mice, as we have observed it (2), is essentially a disease of young animals, which become infected either *in utero* or by contact shortly after birth. Mice infected in either way may carry the virus in the blood for considerable periods of time after recovery. According to observations made in 1935 the number of carriers in the infected stock decreased with increasing age (2). In tests made late in 1937, however, all the mice tested, young and old alike, were carriers.

In the following experiments an attempt was made to determine whether the mode of natural infection had any influence on the duration of the infection in the animals.

Seven pregnant mice 4 to 5 months of age, looking healthy and vigorous, and known to carry virus in the blood, were removed from the infected stock, each animal being placed in a small breeding cage. 2 young ones were taken from each litter immediately after birth and their brains suspended, each in 2 cc. saline. The suspensions were tested for virus by intracerebral inoculation of 2 mice with 0.05×10^{-3} cc. Virus was detected in every case, and the litters were therefore assumed to have become infected *in utero*. It was known from two previous experiments that in litters infected in this manner all the young are infected when born.

The young mice infected *in utero* showed no definite signs of illness except a slightly decreased growth rate in comparison with mice of the same age born from normal mothers. In no instance did these show the severe disease frequently seen in mice infected *in utero* in 1935 (2).

When the 7 litters just mentioned (marked A to G in Text-fig. 1) were 17 to 23 days of age, a new-born normal litter together with the mother was exposed to each of them. The exposed litters marked H to N are recorded in Text-fig. 2. Some of the young of these litters were devoured by the other mice during the first days of life, but enough animals remained to continue the experiment. To determine whether the exposed young mice had become infected by contact with those infected *in utero*, 1 young of each litter was killed on the 10th day after exposure and a suspension of the brain in 2 cc. saline was tested for virus by intracerebral mouse injection. The brains of 5 of the 7 young mice tested contained

TEXT-FIG. 1. Persistence of virus in the blood of mice infected *in utero*.

TEXT-FIG. 2. Persistence of virus in the blood of mice infected by contact shortly after birth.

⊗ = Heart blood infectious.
○ = Heart blood not infectious.
⬤ or ⬤ = Mouse died from injury through heart puncture.
-⊗- = Mouse died naturally some time after bleeding.
* = Highest decimal dilution producing characteristic symptoms on intracerebral injection in a mouse.
** = Titration endpoint not reached at this dilution.
i = Completely immune to intracerebral injection with virus.

virus. Those from litters K and M gave a negative result, but these litters nevertheless became infected as shown by the results of later blood tests.

The severity of the resulting disease indicated by the number of + signs in the text-figures varied with different litters. The mice of litters I, J, K, L, and M contracted a severe disease and several of them died, while those of litters H and N showed a less severe reaction, a slightly decreased rate of growth (marked by one +) being the only sign of disease in litter H. All of the sick mice had completely recovered by the end of the 5th week and later looked like normal, uninfected animals. At the age of about 10 months several mice of litters A to N began to show signs of old age, such as lack of liveliness, adiposis, and ruffling of the fur. On the whole, the animals appeared to age sooner than uninfected mice.

The mice of the corresponding litters (A and H, B and I, and so on) were kept together until the contact litters were 3 to 4 weeks old, when the males and females of each litter were separated. The mice of litters A to G, exceeding in numbers those of the corresponding contact litters, were removed and tested for immunity by intracerebral inoculation with virus. They all proved completely resistant and were then discarded.

The blood of the mice of litters A to N was tested for virus for the first time at the age of 3 to 4 weeks, when all the animals carried virus in the circulation. The results of the later tests for virus and immunity are recorded in the text-figures and need not be reviewed in detail. The mice tested for immunity were completely resistant as evidenced by the absence of clinical symptoms and brain lesions after the intracerebral test injections.

The results given in the text-figures show that the mice infected *in utero* carry virus for a longer period and in greater amounts than do those infected by contact. The great regularity in the persistence of virus in the blood of mice infected *in utero* (Text-fig. 1) is unrelated to the severity of the disease, which plays an important part in mice infected experimentally, as will be shown later. In the mice infected by contact shortly after birth (Text-fig. 2) the severity of the reaction likewise did not seem to influence the persistence of virus to any great extent. In another experiment, however, in which 2 newborn normal litters were exposed to suckling mice that had been injected intranasally with virus 6 days previously, the time of persistence of the virus appeared to be in proportion to the severity of the disease contracted by the exposed animals, as is shown in Table I. The blood of these mice was tested for virus 2½ months after exposure.

Virus Content of Blood Fractions.—In previous tests made with blood from carrier mice the blood cells washed 3 times in large amounts of heparinized saline were either devoid of infectivity or

contained very little virus in comparison with the plasma. Similar observations were made with the blood of animals taken during the acute stage of the disease (3, 4). In three more recent tests with carrier blood the washed cells contained considerable amounts of virus, which in two experiments amounted to about one-tenth of the virus content of the plasma, while in the third test the washed cells and the plasma were about equally virulent.

TABLE I

Influence of the Severity of the Disease on the Persistence of Virus in Suckling Mice after Contact Infection

Litter No.	Mouse No.	Severity of disease	Result of test for virus in blood (2½ months after exposure)
1	1	+++	Positive
	2	+++	"
	3	+++	"
	4	+	"
	5	+	"
	6	0	Negative
	7	0	"
	8	0	"
2	9	+++	Positive
	10	+++	"
	11	+++	"
	12	+	"
	13	+	"
	14	+	Negative
	15	+	"
	16	±	"
	17	±	"
	18	±	"

Distribution of Virus in the Body of Carrier Mice.—It was of interest to determine whether the blood of carriers contained the bulk of the virus present in the body, or whether the high virus content of certain organs was responsible for the virus present in the circulation.

In the experiments presented in Table II the virus content of the blood was compared with that of certain tissues, and it can be seen that almost all the organs tested contained more virus than could be

TABLE II

Comparison of Virus Content of Blood and Organs of Old Carriers

Mouse No.	Date of test	Titer													
		Blood	Nasal turbinates	Submaxillary salivary glands	Lungs	Brain	Kidneys	Ovaries	Uterus	Testicles	Liver	Spleen	Submaxillary lymph nodes	Bronchial lymph nodes	Inguinal lymph nodes
	1937														
5, litter N	Dec. 13	10^0	?*	10^{-1}	10^{-1}	10^{-2}	$10^{-3}+$†	10^{-1}	10^{-2}		10^{-1}	10^{-1}			
2, " K	" 28	10^0	10^{-2}		10^{-2}		$10^{-3}+$								
	1938														
3, " N‡	Feb. 24	10^{-3}	$10^{-6}+$	$10^{-6}+$	$10^{-6}+$	$10^{-6}+$	$10^{-6}+$	$10^{-6}+$	$10^{-6}+$		$10^{-6}+$	$10^{-6}+$	$10^{-6}+$		
1, " G	Mar. 25	10^{-3}	10^{-5}	10^{-6}	10^{-6}	10^{-7}	10^{-6}			10^{-6}	10^{-5}	10^{-6}	$10^{-7}+$		
1, " M	Apr. 8	10^{-4}	10^{-7}				10^{-6}			10^{-6}			10^{-5}	10^{-5}	$>10^{-6}$

* The injected mice died of bacterial infection.

† Titration end-point perhaps not reached at this dilution.

‡ This animal showed beginning lymphatic leukemia at autopsy, the submaxillary lymph nodes being as large as small peas.

accounted for by their blood content. It is possible therefore that the virus in the blood comes from the infected tissues with which the blood comes in contact during circulation.

As reported before (1, 2) virus can be demonstrated with ease in the urine and nasal washings of naturally infected virus carriers. The same is true in the present experiment as is shown in Table III.

TABLE III

Presence of Virus in Urine and Nasal Washings from Old Carrier Mice

Mouse No.	Date of test	Result of pad injection in guinea pigs			
		Urine		Nasal washings	
2, litter A	*1937* Nov. 2	10^0	D14*	10^0	D 14
	" 9	10^{-1}	D 15	10^{-1}	D 13
		10^{-2}	D 15	10^{-2}	D 14
		10^{-3}	D 27	10^{-3}	D 17
2, " B	Nov. 2	10^0	F, no S, I†	10^0	F, no S, I
	" 20	10^{-2}	" "	10^{-2}	" "
		10^{-3}	" "	10^{-3}	" "
3, " H	Nov. 2	10^0	" "	10^0	" "
	1938 Jan. 17	10^0	" "	10^0	" "
2, " I	*1937* Nov. 2	10^0	" "	10^0	" "

* The inoculated guinea pig died in 14 days.
† The guinea pig showed fever, but no definite symptoms, and was immune to reinoculation with virus.

The virulence of the materials for guinea pigs varied considerably as in the previous experiments. Mouse 3, litter H, for instance, was presumably infected with the same strain of virus as mouse 2, litter A, to which it was exposed, and yet the virus discharged by the 2 mice differed markedly in virulence for guinea pigs. The consistency with which the virus from mouse 2, litter A, killed guinea pigs, and the strains from the other mice induced mild, febrile reactions supports the theory advanced before (5) that the differences observed

are mainly due to variations in the virulence of the virus and not to differences in the susceptibility of guinea pigs.

The virus present in the nasal secretions possibly originated in the nasal turbinates which contained considerable amounts of virus compared with the blood (see Table II), and the high virus content of the urine may have been due to that of the kidneys. Since the salivary glands and testicles were rich in virus, it can be assumed that the secretions of these glands may play a part in the transmission of the virus in exceptional cases. The relatively high virus content of the ovaries and uterus may be important for the infection of embryos, which so frequently occurs in pregnant females carrying virus.

The secretion and excretion of large amounts of virus, which occur over a long period of time, suggest that the virus continues to multiply in the tissues of carriers. If this were not the case, one would expect the virus content of certain tissues, for instance the kidneys and nasal turbinates, to become exhausted in due time. The multiplication of virus does not seem to be associated with an extensive breakdown of infected cells, since necrotic lesions are not frequent in the tissues of chronically infected mice. The lesions detected (1) are mostly inflammatory changes, in which mononuclear cells, particularly lymphocytes, predominate.

Virus Content of Mouse Blood during the Acute Stage of the Disease

A comparison has been made of the virus content of the blood of carrier mice with that of mice infected naturally or experimentally and bled during the acute stage of the disease.

The mice infected *in utero* (Table IV) came from 3 litters born in the infected colony. Mice 1 to 5 were bled on the 1st day of life, mouse 6 on the 5th day, and mice 7 to 10 on the 6th day. None of the mice appeared ill, but the growth rate of their litter mates not bled seemed slightly decreased in comparison with that of normal mice of the same age.

The mice infected by contact were 1 day of age when exposed to new-born mice injected intranasally with virus (mice 1 to 4) or to suckling mice infected *in utero* (mice 5 to 7). The mice were bled on the 17th (mice 1 to 3 and 5 to 7) or 21st (mouse 4) day of exposure, when they showed symptoms of the disease. Mouse 3 was bled twice, first on the 17th day, when it was only slightly sick, and again on the 21st day, when it appeared very ill.

Of the 4 new-born animals injected intranasally with virus, mice 1 to 3 were

bled on the 10th day after inoculation, and mouse 4 on the 13th day. The animals were sick at the time of bleeding.

The mice inoculated intravenously with virus were bled 6 to 8 days after injection, when they showed symptoms of the disease, and the blood of the mice

TABLE IV

Virus Content of Mouse Blood during the Acute Stage of the Disease

Mode of infection and age of mice	Maximum dilution of heart blood producing typical symptoms									
	Mouse No.									
	1	2	3	4	5	6	7	8	9	10
Intrauterine infection. Blood tested for virus at age of 1–6 days	10^{-4}	10^{-5}	10^{-4}	10^{-3}	10^{-5}	10^{-4}	10^{-4}	10^{-3}	10^{-5}	10^{-3}
Contact infection. Exposure to infected mice at age of 1 day. Blood tested for virus at age of 17–21 days	$10^{-5}+$*	10^{-4}	10^{-2} 10^{-5}	10^{-5}	10^{-5}	10^{-4}	10^{-4}			
Intranasal instillation of virus at age of 1 day. Blood tested 10–13 days later	10^{-4}	10^{-4}	10^{-6}	10^{-3}						
Intravenous inoculation in 5-wk.-old mice. Tested 6–8 days after injection	$>10^{-3}$	$>10^{-3}$	10^{-4}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Intracerebral inoculation in 5-wk.-old mice. Tested 7–9 days after injection	$>10^{-3}$	$>10^{-3}$	$>10^{-3}$	10^{-2}						

* $+$ = titration end-point not reached at this dilution.

inoculated intracerebrally was taken on the 7th to 9th day, when they showed characteristic convulsions.

The stock strain of virus described below was used for all experimental infections in this experiment. Blood from anesthetized suckling mice was obtained by heart puncture, 0.02 or 0.05 cc. blood (according to the size of the animals) being

withdrawn with a 0.25 cc. syringe and a 27 gage needle. The blood was mixed immediately with 1.98 or 4.95 cc. heparinized saline, and from this 10^{-2} dilution further dilutions were made. This method is not accurate, but colorimetric tests showed the inaccuracy to be negligible in view of the crude method of titration used. The highest decimal dilution producing typical symptoms in a mouse injected intracerebrally was regarded as the titration end-point.

The suckling mice infected *in utero* or by contact are most suitable for a comparison with the carrier mice recorded in Text-figs. 1 and 2. This shows that the average virus content of the blood (34,300 M.I.D. per 0.05 cc. heart blood) of the 10 mice infected *in utero* (Table IV) is only about twice as great as that of the old carriers (19,000 M.I.D. per 0.05 cc. blood) infected in the same manner and recorded in Text-fig. 1. Of the carriers only those animals were considered in which the titration end-point of the blood was reached. With the mice infected by contact soon after birth the difference in virus content between the chronically and acutely infected animals is much greater.

In the groups of suckling mice infected either by contact or by intranasal instillation of virus (Table IV) the virus content of the blood was greater in animals that were very sick than in those which showed a comparatively slight reaction, and the same was·true for the mice injected intravenously. The blood of the mice inoculated intracerebrally with virus was much less infectious than that of the mice infected in other ways. The reason for this difference is not known.

The Influence of the Mode of Experimental Infection on the Persistence of Virus in the Blood of 5 to 6-Week-Old Mice

The three strains of virus used were identical immunologically but differed in their pathogenicity for mice.

The "stock" strain has been maintained by natural passage in the infected stock since 1934. The thoracic and abdominal organs of suckling mice were used as sources of virus. All of the normal mice injected intracerebrally with such virus showed characteristic symptoms 6 to 8 days after inoculation, but the rate of mortality varied between 50 and 100 per cent with different virus suspensions. The majority of the mice inoculated intravenously or intraperitoneally became sick, the mortality varying within wide limits. Subcutaneous inoculation always failed to cause symptoms, but was followed by circulation of

virus and immunity to intracerebral injection with virus. Intranasal instillation[1] rarely produced symptoms. The disease so induced was never fatal and immunized mice against subsequent intracerebral inoculation with virus.

The second strain was the guinea pig passage strain B described before (5) and maintained by serial pad passages in guinea pigs, using brain suspension as inoculum. The virus had undergone 15 to 18 serial transfers. Its virulence for mice is comparable to that of the stock strain, except that it more often causes disease after intranasal instillation.

The third strain was the mouse passage strain B also mentioned before (5). It was modified by serial brain-to-brain transfers in mice. The virus used in the present experiments had been passed through 40 to 45 mice. It is highly virulent when injected intracerebrally but has failed to produce symptoms after inoculation by other routes. Intravenous, intraperitoneal, subcutaneous, or intranasal injection was always followed by immunity, while a small percentage of the mice inoculated intranasally failed to become resistant.

A summary of the data on the pathogenicity of the three strains is given in Table V.

In the following experiments a number of mice were injected by different routes with each strain, and their blood was tested for virus at various periods of time after inoculation. In order to correlate the virus content of the blood with the immunity, the mice that survived the disease as well as the bleedings were tested for immunity 2 to 3 weeks after the blood test, except one group of mice inoculated intravenously with mouse passage virus and tested for immunity on the 6th day, immediately after the blood test. A suitable number of normal control mice was used in every case.

The inoculum consisted of the supernatant of a 10 per cent suspension, centrifuged for 5 minutes at about 1600 R.P.M., of the thoracic and visceral organs of suckling mice from the infected colony (stock strain), the supernatant of a 10 per cent guinea pig brain suspension (guinea pig passage strain), or the supernatant of a 10 per cent mouse brain suspension (mouse passage strain). The dosage on intravenous, intraperitoneal, or subcutaneous inoculation was 0.25 cc., that on intranasal instillation 0.05 cc. Since homologous brain extract is very toxic when injected intravenously in mice, the mice inoculated by this route with the mouse passage strain received 0.25 cc. supernatant of a 10 per cent suspension of the brain of a guinea pig that was killed during fever on the 6th day after intracerebral inoculation with mouse passage virus.

The essential details of the experiment are presented in Table VI which shows the marked differences obtained with different modes of

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[1] 0.05 cc. supernatant of a tissue suspension was dropped on the nostrils of an anesthetized mouse with a 0.25 cc. syringe and a 27 gage needle. The material was promptly inhaled, and the results obtained by this method were much more consistent than those of the procedure employed before (6).

infection and different strains of virus. It can be seen that the duration of the infection in general corresponded to the severity of the disease. The more severe the reaction of the mice was, the longer the virus persisted in their blood.

The results obtained with different strains of virus, however, are not entirely explained by the differences in the severity of the disease induced by them. Subcutaneous inoculation of the guinea pig passage virus, for instance, failed to cause a visible reaction in any of the

TABLE V

Virulence of Three Strains of Choriomeningitis Virus for 5 to 6-Week-Old Mice

Strain of virus	Route of inoculation				
	Intra-cerebral	Intrave-nous	Intraperi-toneal	Subcuta-neous	Intranasal
Stock	+ (50–100)*	± (0–60)	± (0–43)	− (0)	∓ (0)
Guinea pig passage	+ (70–80)	+ ?†	+ (20)	− (0)	± (0)
Mouse passage	+ (80–100)	− (0)	− (0)	− (0)	− (0)

+ = all injected mice became sick.
± = over 50 per cent of the injected mice became sick.
∓ = less than 50 per cent of the injected mice became sick.
− = all injected mice showed no symptoms.
* The percentage of mice that died is given in parentheses. The mice inoculated intracerebrally received more than 10 M.I.D. of virus. Smaller amounts often produce inapparent infection followed by immunity.
† The number of mice injected intravenously with guinea pig passage virus was too small to determine the average rate of mortality.

12 mice injected but created a high percentage of carriers. The mouse passage strain, on the other hand, was not demonstrable in the blood of 6 mice recovered from severe convulsions caused by intracerebral infection and bled about 1 month after inoculation (these tests are not recorded in the table; they were made whenever mice recovered from intracerebral infection were available). It seems therefore as if certain properties of the virus distinct from its pathogenicity accounted for these results.

TABLE VI

Persistence of Virus in the Blood of 5 to 6-Week-Old Mice Injected by Different Routes with Different Strains of Virus

Strain of virus	Route of inocu- lation	Severity of disease	Test for virus in blood			Degree of immunity		
			Time after injec- tion	Virus carriers		Com- plete	Partial (accel- erated, non- fatal reac- tion)	None
				No.	Per- cent- age			
			days					
Stock	iv	++ (1)*	160	9/15†	60	14/14†	0	0
	ip	+++ (3)	160	7/12	58.3	10/10	0	0
	in	— (0)	160	2/14	14.3	9/13	4	0
	sc	— (0)	160	0/12	0	0/12	12	0
"	ip	± (0)	60	2/13	15.4	9/12	3	0
	in	— (0)	60	0/12	0	5/10	4	1‡
	sc	— (0)	60	0/12	0	1/11	10	0
Guinea pig passage	ip	++ (4)	60	9/12	75	10/10	0	0
	"	+ (0)	60	3/8	37.5	8/8	0	0
	sc	— (0)	60	8/12	66.7	9/10	1	0
Mouse passage	iv	— (0)	45	0/13	0	0/13	12	1‡
	ip	— (0)	6	7/12	58.3	11/11	0	0
	"	— (0)	30	0/12	0	9/10	1	0
	in	— (0)	30	0/10	0	5/9	2	2§

iv = intravenously.
ip = intraperitoneally.
in = intranasally.
sc = subcutaneously.
* The number of mice that died from the disease is given in parentheses.
† The numerator represents the number of mice with either circulating virus or complete immunity; the denominator indicates the number of mice tested.
‡ Dead on the 2nd day after intracerebral inoculation.
§ " " " 7th " " " "

Tests made from time to time with the urine and nasal washings of some of the mice recorded in Table VI, using guinea pigs as test animals, gave results similar to those of the blood tests.

The different degrees of immunity observed in the mice require some explanation. If an animal showed no visible reaction whatever

to the intracerebral test injection during a 2 weeks' period of observation, its immunity was considered as complete. "Partial immunity" manifested itself in an accelerated but non-fatal reaction to the test inoculation. Such partially immune mice would show marked symptoms of malaise, inappetence, loss of weight, ruffled fur, as well as striking tremors and jerky motions of the hind extremities when lifted by the tail, on the 2nd or 3rd day after the test injection. Convulsions have not been noted, and the animals as a rule made a quick recovery, appearing practically normal on the 5th or 6th day after inoculation, when the normal controls injected in the same manner began to show the first symptoms. Histological examination of the brains of mice killed during the accelerated reaction on the 2nd day after inoculation revealed a marked meningo-encephalitis, which could hardly be distinguished from that presented by control mice killed at the height of the disease on the 6th or 7th day after injection, except that the tendency to cerebral hemorrhages was greater in the former animals. The brains of control mice killed on the 2nd day after inoculation showed only traces of meningitis, if any. It is believed that the accelerated reaction is due to the allergic state of the mice with a waning immunity.

As can be seen from Table VI, the number of partially immune, allergic mice was greatest in those groups that contained few or no carriers, while the mice of other lots with a high percentage of carriers as a rule were completely immune. Particularly noteworthy is the fact that all of the mice with a partial immunity gave a negative blood test.

This could make it appear that the immunity of mice depended upon the presence of active virus in the body. However, numerous other observations do not justify this conclusion. In the first place, we have repeatedly encountered mice with a high degree of resistance in whose bodies no virus was detected (1). The problem was reinvestigated recently, when the results given in Table VI were obtained, and the experiments performed provided additional evidence suggesting that there is an immunity independent of the presence of active virus in the organism. For instance, when mice that had shown a severe accelerated reaction after the intracerebral test injection with mouse passage virus were reinoculated with the same

strain by the same route 1 month later, they failed to show any re-
action. Some such mice killed 16 days after the last inoculation
carried no demonstrable virus in their brains or in any of their blood
fractions. It is unlikely that the presence of virus was masked by
antibodies, since these were either absent from the blood and from a
concentrated brain extract, or possibly present in very small amounts,
which could hardly have been effective. Other tests gave similar
results.

The Influence of Age on the Persistence of Virus in the Blood after Intranasal Instillation

It has been reported (7, 4, 6) that intranasal instillation of chorio-
meningitis virus does not cause illness in mice but immunizes them
to intracerebral injection with virus. In more recent experiments,
however, it has been found that a symptomless infection occurs only
in mature mice (5 weeks of age or older) inoculated intranasally with
certain strains of virus, while immature mice as a rule become sick
and often die.

Some of the inoculations are recorded in Table VII to show the
difference in reaction of mice of different ages. Suckling mice re-
ceived 2 drops of the supernatant of a virulent 10 per cent mouse or
guinea pig brain suspension through a 27 gage needle; 2 to 3-week-old
mice received 3 drops; while the mice 5 weeks of age or older were
given 0.05 cc.

The stock strain caused no signs of illness in the great majority of
the mature mice, while young mice as a rule became sick and many of
them died. The guinea pig passage virus was the most virulent and
even produced a relatively mild, non-fatal disease in mature mice.
Intranasal instillation of the mouse passage virus was effective only
in very young animals.

The symptoms produced in mature mice were those of general
malaise and labored respiration. They did not point to an involve-
ment of the central nervous system. The same was generally true
for immature animals, which showed visceral lesions without meningi-
tis or with only traces of it. A few of the more than 100 young mice
injected, however, showed characteristic convulsions that are other-
wise seen only in mice injected intracerebrally. In 2 to 3-week-old

mice the guinea pig passage virus frequently produced serous pleuritis ending in respiratory failure.

A number of mice of different ages injected intranasally with stock or mouse passage virus were kept for a period of time after recovery and then tested for circulating virus. All mice, including those very sick for several weeks after inoculation, had completely recovered at

TABLE VII

Susceptibility of Mice of Different Ages to Intranasal Injection with Virus

Strain of virus	Age of mice	No. of mice injected	Result		
			No. of mice which		
			Showed no definite symptoms	Became sick and recovered	Died
Stock	1 day	18	0	13 (+++)*	5
	2–3 wks.	12	4	4 (++)	4
	5 "	12	12	0	0
	6–7 mos.	12	12	0	0
	2 wks.	14	0	5 (+++)	9
	5 "	14	3	11 (±)	0
	2 "	26	0	19 (+++)	7
	5 "	9	8	1 (+)	0
Guinea pig passage	1 day	16	0	2 (+++)	14
	7 days	14	0	4 (+++)	10
	2–3 wks.	20	0	7 (++)	13
	5 "	10	0	10 (+)	0
	9–10 "	10	0	10 (+)	0
Mouse passage	1 day	41	3	9 (+++)	29
	12–14 days	12	12	0	0
	5 wks.	12	12	0	0

* The number of + signs indicates the average severity of the disease.

the time of the blood tests and could not be differentiated from uninfected animals.

The results recorded in Table VIII show that the duration of the carrier state differed markedly with the age at the time of injection. The younger the mice were, the longer they carried virus in the blood. Very young mice infected with the stock strain became carriers more frequently than those infected with mouse passage virus.

The Influence of Age on the Persistence of Virus after Contact Infection

The disease induced in mice of different ages by contact infection is similar to that produced by intranasal instillation of virus in that very young mice often become sick, while the disease in mature ones invariably is subclinical. The duration of the infection in mice infected by contact is also comparable to that in mice injected intranasally with virus. As has been shown in Text-fig. 2, many young mice infected by contact carry virus in the blood for long periods of time,

TABLE VIII

Persistence of Virus in the Blood of Intranasally Injected Mice of Different Ages

Strain of virus	Age of mice when injected	Severity of disease	Test for virus in blood		
			Time after injection	Mice with virulent blood	
				Number	Percentage
			days		
Stock	1 day	+++	160	12/12	100
			250	6/6*	100
	1 wk.	+++	160	7/7	100
	5 wks.	±	160	2/14	14.3
"	1 day	+++	60	13/13	100
	2–3 wks.	++	60	5/8	62.5
	5 "	−	60	0/12	0
Mouse passage	1 day	+++	60	4/7	57.1
	12–14 days	−	30	0/8	0

* These 6 mice were among the 12 animals tested on the 160th day after inoculation.

whereas in mature mice infected by contact the virus circulates for 3 weeks at the most. This can be concluded from the data on other experiments on contact infection.

DISCUSSION

Among the factors influencing the persistence of virus after natural infection the age of the mice appears to be the most important one. The more immature the mouse tissues are at the time of infection, the more regularly the virus persists in them. This is indicated by

the fact that the virus remained longer or in greater amount in the blood of mice infected *in utero* than in those infected by contact during the first 3 weeks of life. The severity of the disease, which appears to be the determining factor in mature mice infected experimentally, was of minor importance compared with the age factor.

In the case of the mice of different ages infected by contact or by intranasal instillation of virus the age at the time of infection may likewise be important, but its effect can be less clearly differentiated from that of the severity of the disease, which was greatest in very young mice. These continued to carry the virus for longer periods of time than older mice that showed a slighter reaction.

In some groups of suckling mice infected by contact, as well as in mature mice infected experimentally, the duration of the infection corresponded to the severity of the symptoms which the infected animals had shown. The parallelism recalls the experiments of Beller and Biermann (8) with hog cholera virus, in which swine vaccinated by the simultaneous method discharged virus with the urine longer when they had shown a severe reaction than when the reaction had been entirely absent or very mild.

The differences in the persistence of strains of virus may be due to a combination of two factors, namely, the severity of the disease produced, and a property of the virus itself distinct from its pathogenicity. This is also suggested by the comparatively small percentage of carriers detected among the mice inoculated intranasally with the mouse passage virus during the first 12 days of life, which is in contrast to the high percentage of carriers created by the stock strain.

The high virus content of the blood and certain organs of carrier mice, particularly those infected *in utero*, is of interest with regard to the mechanism of their immunity. It is very unlikely that protective antibodies play an essential part in this immunity, because the average virus content of the blood of carriers infected *in utero* taken at the age of about 1 year is only slightly less than that of mice similarly infected and bled during the 1st week of life. If antibodies were fixed in the tissues, as Bedson (9) suggested, one would not expect to demonstrate such great amounts of virus in them as have been detected. It rather appears that the susceptible cells

of the body are infected in such animals, and that cells once infected with virus cannot be reinfected. This assumption is supported by the fact that new-born mice infected *in utero* are already immune to intranasal instillation of highly virulent virus, which is fatal to many of the new-born control mice and produces a severe disease in those which survive.

The observations of Hoskins (10) and of Findlay and MacCallum (11) on the interference phenomenon in yellow fever are of great interest in this connection. These authors have shown that monkeys infected simultaneously with viscerotropic and neurotropic yellow fever virus often survive, while monkeys inoculated with viscerotropic virus alone almost always die. This effect could even be demonstrated when the neurotropic virus was given up to 20 hours after the viscerotropic strain, and did not appear to be due to a precocious formation of antibodies (11). It seems therefore that cells once infected by the neurotropic virus are refractory to the viscerotropic strain. It is also interesting to note the parallelism between the immunity of carrier mice to choriomeningitis and that of plants to tobacco ring spot virus. Immune plants are always carriers of virus, although they show no signs of disease, but their virus content is about 5 times less than that of diseased plants that show symptoms (12).

If no other data were available than those given in the text-figures and in Tables II and VI, one might conclude that the immunity of mice to choriomeningitis was always an infection immunity. However, some observations reported before (1), as well as the results obtained with mice injected repeatedly with mouse passage virus, in whose blood and brains no virus was demonstrated, fail to justify this conclusion. The mechanism of the immunity of such mice has recently been investigated. While the sera of some animals may possibly have contained minute amounts of protective antibodies, the results obtained with other sera as well as with brain extracts from immune mice were negative. It is tentatively concluded for the present that the immunity of such mice is not an infection immunity, nor due solely to protective antibodies circulating in the blood or fixed in the tissue spaces. It rather appears to be linked in some way with the tissues.

It remains for future studies to determine whether the two kinds of immunity observed really differ, or whether the same fundamental mechanism underlies both of them.

SUMMARY AND CONCLUSIONS

Mice infected *in utero* continued to carry choriomeningitis virus in the blood more regularly and in greater amount than suckling mice infected by contact. This result may be due to the difference in tissue maturity at the time of infection: the more immature the tissues are when infected, the longer the virus appears to persist in them after maturation. A similar result was obtained with mice of different ages infected either by contact or by intranasal instillation of virus, in that the carrier state lasted longer in the younger animals. This cannot be attributed entirely to the difference in age, however, since young mice as a rule showed more severe symptoms than mature animals. It is possible, therefore, that the difference in the severity of the disease accounted in part for that in the duration of the infection.

In mature mice infected experimentally as well as in some of the suckling mice infected by contact the severity of the disease was the determining factor, the infection persisting longest in those animals that showed the most severe reaction.

The character of the virus used also appeared to influence the persistence of the virus in the blood. A strain of virus isolated in 1935 from an infected stock mouse and modified by intracerebral passage in mice (5) disappeared from the circulation more rapidly than the stock strain maintained by natural passage in the infected mouse stock. The guinea pig passage strain, however, which was obtained from the same mouse as the mouse passage virus but passed through guinea pigs by pad inoculation, persisted in the blood more frequently than the stock strain.

Carrier mice without exception had a high degree of immunity to intracerebral injection with virus, while other animals once infected but no longer carrying detectable amounts of virus in the blood often showed an incomplete immunity that manifested itself in an accelerated, non-fatal reaction, presumably of an allergic nature. This observation does not prove, however, that the immunity always is an

"infection immunity," since a high degree of resistance not associated with detectable amounts of virus in the blood and brain was produced by repeated injections with the mouse passage strain.

Since the blood and the tissues of old carriers often contain large amounts of virus, it is very unlikely that their immunity is due to protective antibodies circulating in the blood or fixed in the tissue spaces. It rather appears that the susceptible cells of such animals are infected and that cells occupied by actively multiplying virus cannot be reinfected. The mechanism of this infection immunity as well as the immunity apparently not associated with infection requires further study.

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THE PASSAGE OF RABBIT VIRULENT TYPE III PNEUMOCOCCI FROM THE RESPIRATORY TRACT OF RABBITS INTO THE LYMPHATICS AND BLOOD

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Previous experiments (1, 2) have shown that if rabbits were given large intravenous injections of Type III pneumococci, strain SV (Tillett, 3), or if given blood infections by small intravenous injections, the organisms could be cultivated from thoracic duct, cervical, and leg lymphatics and that intravenous treatment with antisera, while often effective in immediate sterilization of the blood, did not produce the same favorable result in the lymph. The bearing of these experiments on the serological treatment of such infections was quite obvious. It was also shown (4) that a variety of visible particles readily made their way from blood to lymph. It thus became a matter of interest to see whether pneumococci placed upon the uninjured mucosa of the respiratory tract succeeded in penetrating the tissues and reaching lymphatics. Four groups of experiments were performed with rabbits, the rabbit virulent Type III Pneumococcus, strain SV, being used in all. The experiments were:

1. Cannulation of thoracic duct and trachea. Culture instilled in trachea.

2. Cannulation of a cervical lymphatic and trachea. Esophagus tied. Culture instilled intranasally.

3. Intravenous injection of antipneumococcus horse serum. Cannulation of thoracic duct and trachea. Culture instilled in trachea.

4. Intravenous injection of antipneumococcus horse serum. Can-

251

nulation of a cervical lymphatic and trachea.   Esophagus tied.   Culture instilled intranasally.

Material and Methods

With but few exceptions, albino rabbits were used throughout the experiments. The animals, averaging 2 kilos, were anesthetized by the intravenous administration of a 5 per cent sodium pentobarbital (nembutal) solution, beginning with an initial dose of 35 mg. per kilo of body weight and adding small amounts subsequently in order to keep the anesthesia uniform.

The cervical lymphatics and thoracic ducts were exposed and cannulated as previously described (1).   In order to be sure that thoracic duct lymph was not contaminated by blood entering the duct through small veins, a circumstance not infrequent in the rabbit, red cell counts were made in all cases, and in the experiments given the red cell content of the thoracic duct lymph never was above normal limits so that it is certain no gross contamination with blood occurred. In the group of animals receiving intranasal instillations the esophagus was tied and the trachea cannulated in order to prevent the animal from swallowing or inhaling the instilled culture.   The right jugular vein and carotid artery were exposed for the taking of blood samples.

Two methods were employed for the culture of blood samples.   One consisted in streaking 0.5 cc. of arterial and venous blood over plates, the other in adding 0.5 cc. of freshly drawn blood to melted nutrient agar as plates were poured. During the early experiments blood was withdrawn every 10 minutes.   In later experiments it was withdrawn at half hour intervals.

Lymph for culture was collected as it accumulated.   A portion of a sample was at once streaked on blood agar plates, a second portion smeared on a slide, a third portion centrifuged and the sediment used in making smears, and a final portion was used for broth cultures.   At the end of the experiment, tissues from the exposed areas were fixed for microscopic study, including the accompanying regional lymph nodes.

The organisms employed were derived from an SV strain of Type III pneumococci which was known to cause the death of rabbits following the intradermal injection of 0.001 cc. of a 16 hour blood broth culture, and from time to time the organisms were passed through rabbits to assure their virulence.   The respiratory tract of each animal received the centrifuged concentrate from 20 cc. of a blood broth culture which had been incubated for 16 hours.   The organisms were resuspended in 2 cc. of the supernatant broth.   Difficulty in retaining a full dose given intranasally was occasionally encountered.   In order to prevent such temporary overloading, the organisms were administered in two doses, one at the beginning of the experiment and the remainder 2 hours later.

Films made directly from lymph and those from concentrated lymph sediment were stained with Wright's blood stain and by Gram's method.

In the third and fourth group of observations antiserum was administered

intravenously 2½ to 3 hours before the organisms were instilled in the nose and in the trachea. The antiserum used had an agglutinating titre of 1:400. The number of organisms recovered from lymph samples is recorded roughly in from one to four plus signs for each half hour period.

RESULTS

Tables I and II show that the organisms were first usually demonstrated in the thoracic duct and cervical lymph in the second half hour specimen. The number of cases in which the lymph became positive increased up to the fourth half hour period when the lymph in the majority of the animals had become positive. In two animals (Nos. 13 and 18) organisms did not appear during the experiment. Variation in the number of organisms recovered at intervals after they were first demonstrated was at times definitely associated with increased and decreased flow of lymph during that period, usually due to clots forming at the tip of the cannula.

It is apparent from Table I that pneumococci in fluid suspension placed in the trachea and frequently reaching the alveoli, as observed at autopsy, made their way into the thoracic duct lymph quite rapidly. In order to do this, lung lymphatics must be traversed and lymph nodes passed at the root of the lung. Similarly (Table II) organisms instilled intranasally soon began to appear in the cervical lymph. In this latter case there must have been penetration of the nasal mucous membrane and passage of at least one cervical lymph node before the point of cannulation was reached low in the neck. The blood rarely became positive even after intratracheal instillation, and in the intranasal group the blood was practically free from organisms during all experiments. Table III, covering 10 rabbits which received antiserum and then, after several hours, pneumococci intratracheally, shows a few positive results for organisms in thoracic duct lymph, but evidently the antiserum has had some effect in controlling lymph infection. In Table I, presenting comparable data for 19 rabbits similarly infected but unprotected by antiserum, there are 16 in which the thoracic duct lymph became positive, whereas in Table III, composed of data upon 10 animals given antiserum, only 4 showed organisms in the thoracic duct lymph. In a similar manner it is apparent that antiserum has been of assistance in connection

TABLE I

No.	Weight (kg.)	Temperature (°C.)	Red blood cells per c.mm. lymph	0.5 hr. T	0.5 hr. B	1.0 hr. T	1.0 hr. B	1.5 hr. T	1.5 hr. B	2.0 hr. T	2.0 hr. B	2.5 hr. T	2.5 hr. B	3.0 hr. T	3.0 hr. B	3.5 hr. T	3.5 hr. B	4.0 hr. T	4.0 hr. B
1	2.2	38.0	600	+	++++	+	++++	+	++++	++	++++	++	++++	+++	++++	++	++++	++	+++
2	1.8	38.5	None	0	0	0	0	0	0	0	0	+	0	++	0	+	0	Died	Died
3	1.9	39.0	None	0	0	+	0	++	0	+	0	++	+	+++	0	+++	0	++++	++
4	2.0	39.0	100	0	0	0	0	++	0	+	0	++	0	+++	+	++	0	++	+
5	1.7	40.0	None	0	0	0	0	0	0	0	0	++	0	++	0	++	0	+++	0
6	2.0	39.5	None	0	0	0	0	+	0	++	0	+	+	+	0	+	0	+++	+
7	2.0	40.0	200	0	0	0	0	+	0	++	0	+	0	0	0	0	0	+++	0
8	2.1	39.0	None	0	0	+	0	0	0	++	0	++	0	+	0	++	0	0	0
9	2.0	39.5	300	0	0	0	0	+	0	++	0	++	0	++	0	++	0	++	+
10	1.8	40.0	None	0	0	0	0	0	0	0	0	+	0	+	0	+	0	0	0
11	1.6	39.5	200	0	0	0	0	0	0	0	0	++	0	+	0	++	+	+++	0
12	2.0	40.0	200	0	0	0	0	+	0	+	+	+	0	+	0	++	0	++	0
13	2.8	39.0	100	0	0	0	0	Died	Died	0	0	0	0	0	0	+	0	++	+
14	1.4	40.0	200	0	0	+	0	+	0	0	0	0	0	0	0	0	0	0	0
15	2.0	39.2	200	0	0	0	0	0	0	++	+	++	+	0	0	+	+	+++	+
16	4.6	40.1	None	0	0	0	0	+	0	+	0	+	0	+	0	+	0	0	0
17	2.0	40.0	100	0	0	0	0	0	0	+	0	+	0	+	0	+	0	+	+
18	2.3	40.0	200	0	0	0	0	0	0	0	0	0	0	+	0	+	0	+0	0
19	2.3	41.0	None	0	0	0	0	0	0	+	0	+	+	Died	Died	0	0	0	0

This table shows the results of instilling 2 cc. of rabbit virulent Type III Pneumococcus culture into the trachea of normal rabbits. +, 1 to 5 organisms per half hour sample; ++, 5 to 15 organisms; +++, 15 to 30 organisms; ++++, 30 and above organisms. T, thoracic duct lymph; B, blood.

TABLE II

No.	Weight	Tempera-ture	0.5 hr.		1.0 hr.		1.5 hr.		2.0 hr.		2.5 hr.		3.0 hr.		3.5 hr.		4.0 hr.		
	kg.	°C.	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	
20	1.9	39.0	0	0	0	0	++	0	+	0	0	0	+	0	++	0	++	0	Flow scant
21	2.6	40.0	0	0	++	0	0	0	++	0	0	0	+	0	+	+	+	0	Flow scant
22	1.6	36.0	0	0	0	0	0	0	0	0	0	0	+	0	+	0	+	0	
23	2.7	37.2	0	0	0	0	0	0	+	0	++	0	+	0			++	0	
24	2.0	38.0	0	0	0	0	0	0	+	0	0	0	0	0	0	0		0	Lymphatic dilated above node
25	2.2	38.2	0	0	+	0	0	0	0	0									
26	2.2	38.1	0	0	0	0	+	0	0	0	++	0	++	0	+	0	0	0	
27	2.1	38.0	0	0	0	0	0	0	+	0	Died	0	Died						
28	2.0	37.7	0	0	0	0	0	0	0	0	+	0	+	0					
29	2.0	38.5	0	0	0	0	0	0	0	0	+	0	+	0	+	+	0	0	Flow scant

This table shows the results of instilling 2 to 3 cc. of rabbit virulent Type III Pneumococcus culture into the nose of normal rabbits. +, 1 to 5 organisms per half hour sample; ++, 5 to 15 organisms. C, cervical lymph; B, blood.

TABLE III

No.	Weight (kg.)	Temperature (°C.)	Red blood cells per c. mm. lymph	Time between antiserum injection and instillation of organisms (hrs.)	(min.)	0.5 hr. T	0.5 hr. B	1.0 hr. T	1.0 hr. B	1.5 hr. T	1.5 hr. B	2.0 hr. T	2.0 hr. B	2.5 hr. T	2.5 hr. B	3.0 hr. T	3.0 hr. B	3.5 hr. T	3.5 hr. B	4.0 hr. T	4.0 hr. B
30	2.2	38.0	200	3	20	0	0	+	0	+	+	+	0	+	0	0	0	0	0	0	0
31	2.4	37.5	200	3	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	1.7	38.4	None	2	20	++	++	++	++	++	++	++	++	++	+	+	++	++	++	+	++
33	1.9	37.8	400	2	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	3.7	39.1	None	2	50	0	0	+	0	+	0	0	0	+	0	0	0	0	0	0	0
35	2.6	39.4	None	3	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36	2.7	37.8	None	3	10	0	0	0	0	+	0	+	0	0	0	0	0	0	0	0	0
37	2.2	38.1	200	2	55	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
38	3.3	38.0	100	3	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	2.1	38.2	None	3	00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

This table shows the result of instilling 2 cc. of rabbit virulent Type III Pneumococcus culture into the trachea of rabbits which had received previously 3 to 5 cc. of antipneumococcus horse serum intravenously. +, 1 to 5 organisms per half hour sample; ++, 5 to 15 organisms; +++, 15 to 30 organisms; ++++, 30 and above organisms. T, thoracic duct lymph; B, blood.

TABLE IV

No.	Weight	Temperature	0.5 hr.		1.0 hr.		1.5 hr.		2.0 hr.		2.5 hr.		3.0 hr.		3.5 hr.		4.0 hr.	
		°C.	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B
	kg.																	
40	2.1		0	0	0	0	0	0	0	0	Died							
41	2.7		0	0	0	0	0	0	Died									
42	2.1	38.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
43	2.3	40.0	0	0	0	0	+	0	0	0	+	0	0	0	0	0	0	0
44	2.3	39.0	0	0	0	0	0	0	++	0	+	0	+	0	No flow		0	0
45	2.1	38.0	0	0	0	0	0	0	+	0	Died							
46	1.9	40.0	0	0	0	0	0	+	0	0	++	0	+	0	0	0	+	0
47	1.6	40.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48	2.3	41.5	++	0	++	0	+	0	+	+++	++	0	+++	++++	++	0	+++	+++
49	2.0	39.0	++	0	0	0	+	+	+	0	+	0	+	+	+	0	+++	++++
50	1.9	40.0	0	0	0	+++	0	0	0	0	0	0	0	0	0	0	0	0
51	1.8	40.5	0	0	0	0	0	+	0	0	0	0	0	+	0	0	0	0
52	1.8	40.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
53	1.8	39.0	0	0	0	0	+	0	+	0	0	0	0	0	0	0	0	0

This table shows the results of instilling 2 cc. of rabbit virulent Type III Pneumococcus culture into the nose of rabbits which had received 5 cc. of antipneumococcus horse serum previously. +, 1 to 5 organisms per half hour sample; ++, 5 to 15 organisms; +++, 15 to 30 organisms; ++++, 30 and above organisms. C, cervical lymph; B, blood.

prior to the instillation of organisms in the nose or in the trachea, it is reasonable to believe that the administration of this serum was responsible for the negative cultures of lymph in the animals whose lymph showed a relatively high antiserum titre. Those cases in which there were positive lymph cultures at the onset and which subsequently became negative, coupled with a fair titre of antiserum in the lymph, show more strikingly the beneficial effect of antiserum. It is more difficult to account for the three animals upon which the antiserum did not appear to have any effect. But one of these showed no antiserum in the lymph in any of the specimens examined. Previous work (1, 2) has indicated that it is difficult to obtain concentrations of antisubstances in the lymph of rabbits capable of nullifying infection permanently, but it did not yield instances in which no antisubstances were detectable in lymph.

The penetration of draining lymphatics by organisms placed upon the surface of the nasopharyngeal mucous membrane raises a number of questions. First of all, does one find in control specimens of cervical lymph taken prior to intranasal instillation any of the organisms commonly present upon the nasal mucosa? The answer with very rare exceptions in our experience is "No." On one occasion the cervical lymph of a monkey contained a streptococcus and a pneumococcus, neither of which is a probable contaminant. But this experience stands alone in many experiments upon different species. Furthermore, if one repeats the experiments recounted above, using a non-virulent organism, the lymph remains sterile. The same thing is true when the nasopharyngeal mucosa is flooded with graphite particles of bacterial dimensions. No graphite is found in the lymph. It cannot, of course, be said with certainty that organisms non-virulent for the host fail to make the passage to the lymph, but if they do reach this destination they have become incapable of growth or they would have been detected in cultures. By analogy, with the failure of graphite particles to enter the lymph we think that organisms incapable of multiplying in the host do not survive far past the penetration of the mucous membrane if they even leave the surface at all.

Upon the mechanism of penetration of virulent organisms we have nothing to offer. With tracheal cannulation and the esophagus tied,

there is practically no motion of the nasopharyngeal membrane such as might accompany breathing or swallowing and, as has been pointed out, phagocytosis is not necessary. In the case of the instillations into the lungs, it is more than possible that non-virulent or even dead organisms may reach the lymph stream since graphite particles and particles of other sorts penetrate within an hour to the tracheo-bronchial lymph nodes. Once there and lacking power to multiply vigorously, it would again be difficult for organisms to go further and reach the cannula collecting thoracic duct lymph.

SUMMARY

1. Rabbit virulent Type III pneumococci when instilled into the nose or trachea were recovered from the lymphatics draining the area involved in the lymph collected during a subsequent 4 hour period. Their detection rarely failed, and not infrequently was possible at the end of the 1st hour.

2. The organisms practically invariably appeared first in the lymphatics and subsequently in a few cases were recovered from the blood during the 4 hour test period.

3. The intravenous administration of antiserum $2\frac{1}{2}$ to 3 hours before the instillation of organisms decreased the number of animals whose lymph or blood became positive and the total length of time in which organisms were recovered in lymph from the efferent lymphatics during the test period.

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RELATION BETWEEN DEGREE OF IMMUNITY OF MICE FOLLOWING VACCINATION WITH ST. LOUIS ENCEPHALITIS VIRUS AND THE TITRE OF THE PROTECTIVE ANTIBODIES OF THE SERUM

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It has been shown that the subcutaneous injection of St. Louis encephalitis virus into susceptible mice renders them resistant to subsequent intracerebral and intranasal inoculation (1). The present experiments were designed to study the relation between the degree of immunity produced and the titre of circulating protective antibodies. It was believed that by investigation of this relationship the importance of humoral protective antibodies for immunity might be estimated. Accordingly, susceptible mice were vaccinated and at intervals thereafter their immunity against intracerebrally injected virus was determined and compared with the protective antibody titre of their sera.

Materials and Methods

Mice.—Mice employed in Experiment 1 were of the selected Swiss strain. They are uniformly susceptible to St. Louis encephalitis virus. In Experiment 2 mice of high inherent resistance to the St. Louis virus were used (2). In both experiments control mice were of the same age as the vaccinated mice.

Virus.—The virus used throughout was the St. Louis encephalitis virus, strain 3 (3). Virus suspensions were prepared in the following manner: The brain of a mouse prostrate with encephalitis was removed under sterile conditions and ground in a mortar. The emulsion of brain tissue was then diluted with ten times its weight of hormone broth of pH 8.0. After thorough mixing the suspension was centrifuged at 1,000 R.P.M. for 5 minutes and the supernatant made up in serial tenfold dilutions in broth.

Vaccination.—The mice were immunized by one subcutaneous injection of 0.5 cc. of a 1:1,000 dilution of the virus prepared as described above. Each time the virus was used for vaccination it was titrated by intracerebral injection into

Swiss mice. These titrations showed that the amount of virus used for vaccination was approximately 15,000 times the minimal lethal intracerebral dose. The subcutaneous injection of this quantity of virus did not produce encephalitis nor cause any other symptoms of illness.

Resistance Tests.—At intervals following vaccination the degree of the acquired immunity of the vaccinated mice was estimated by comparing their resistance to intracerebral inoculation of the virus with that of unvaccinated controls. All mice tested were under light ether anesthesia; each received 0.03 cc. of the virus appropriately diluted in broth.

Neutralization Tests.—St. Louis virus infection and immunity in the mouse are chiefly concerned with the susceptible brain tissue for which the virus has an almost specific affinity. Hence interest in the action of the serum of mice immunized by vaccination rests chiefly on its ability to neutralize the injurious effect of the virus upon the brain. To test this action, serum and virus were mixed and injected directly into the brain, employing the technique described below. No more than passing interest was taken in the capacity of the serum to modify the activity of the virus when introduced by routes ordinarily non-pathogenic, such as the intraperitoneal.[1]

All sera used in the neutralization tests were obtained from anesthetized mice by heart puncture. Tests on vaccinated mice were done with serum derived from the pooled blood of six mice. In each test, serum of unvaccinated mice was used as the negative control, and serum of a hyperimmunized monkey was employed as the positive control.

Virus suspensions used in the neutralization tests were prepared in suitable dilutions in the manner described above. 0.3 cc. of each dilution of virus employed was added to 0.3 cc. of undiluted serum. The mixtures of serum and virus were incubated for 2 hours at 36°C. and then left at room temperature for 2 hours. After incubation 0.03 cc. of each serum-virus mixture was injected intracerebrally into each of four mice under ether anesthesia. The inoculated mice were then observed for 21 days for the possible development of encephalitis.

Interpretation of Neutralization Tests.—By making a statistical analysis of the results of neutralization tests carried out in this laboratory with known negative and known positive sera against St. Louis encephalitis virus, Muench (4) established the following criteria: Any serum which fails to protect more than 50 per cent of the injected mice against 1 M.L.D. of the virus is considered negative.

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[1] In this connection it is noteworthy that it has recently been reported (5) that the protective power of antisera against equine encephalomyelitis virus is much greater when serum-virus mixtures are inoculated intraperitoneally into young mice than it is when the intracerebral route is employed. However, serum obtained by us from a small number of mice immunized with St. Louis virus did not show significantly greater protection after intraperitoneal inoculation of serum-virus mixtures than after intracerebral injection.

Sera protecting 75 per cent of the mice against 1 M.L.D. of virus are regarded as doubtfully positive. Any serum which protects completely against 1 M.L.D. of virus is definitely positive. This classification was followed in interpreting the results of the neutralization tests carried out during the experiments described below. It is interesting to note that the result of only one test fell into the doubtful group. All others were definitely positive or definitely negative. If we arbitrarily consider as negative all those sera which fail to furnish complete protection against 1 M.L.D. of virus, we find that the results are practically the same as those arrived at by applying Muench's criteria.

EXPERIMENTAL

Experiment 1.—497 mice 6 weeks of age at the beginning of the experiment were employed. 241 of these animals were put aside as controls, while 256 were vaccinated with virulent St. Louis encephalitis virus. At intervals thereafter, ranging from 2 days to 23 weeks, both their resistance and the neutralizing antibodies in their blood were tested by the methods described above.

Two days after vaccination the mice were unable to survive the intracerebral injection of 1 M.L.D. of virus. After 1 week a second and third group tested showed a very strong immunity to the virus. At this time the vaccinated mice were resistant to 10,000 minimal lethal intracerebral doses. 2 and 3 weeks after vaccination immunity was at this same high level. After 6 weeks the immunized mice still withstood an intracerebral injection of 1,000 M.L.D. of virus. At 8 and 10 weeks, however, immunity had decreased to such a degree that the mice were completely resistant to no more than 10 intracerebral M.L.D. 12 weeks after vaccination the immunity had decreased still further, so that the mice were not uniformly immune against even 1 M.L.D. of virus. At 20 and 23 weeks after vaccination no immunity could be demonstrated.

In brief, the results summarized in Table I indicate that vaccination of mice with St. Louis encephalitis virus induces an immunity which appears within 1 week after vaccination. When first demonstrable, this immunity is at a very high level and it remains so for about 6 weeks, after which it diminishes and disappears between the 12th and 20th week.

The first neutralization test was done with serum obtained from mice 1 day after vaccination. This serum, when mixed with virus and injected intracerebrally into unvaccinated susceptible mice, did

not protect any of them against even 1 M.L.D. of virus. It was therefore regarded as negative. Tests made with two pools of serum 1 week after vaccination were negative. This negative result is in striking

TABLE I

Immunity to Intracerebral Inoculation of St. Louis Encephalitis Virus Following Vaccination

Time after vaccination	Treatment of mice	Mortality of mice injected intracerebrally with St. Louis encephalitis virus							Degree of immunity of vaccinated mice
		Dilution of virus injected intracerebrally							Intracerebral M.L.D.
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
days									
2	Vaccinated	—	—	—	3/3*	3/3	2/3	—	0
	Unvaccinated	—	—	—	3/3	3/3	3/3	—	
wks.									
1	Vaccinated	2/3	—	0/3	—	0/3	—	—	10,000
	Unvaccinated	3/3	—	3/3	—	3/3	3/3	2/3	
1	Vaccinated	—	1/3	0/4	0/4	0/4	—	—	10,000
	Unvaccinated	—	—	—	—	4/4	3/4	0/4	
2	Vaccinated	—	0/4	0/4	0/4	0/4	—	—	10,000
	Unvaccinated	—	—	—	4/4	4/4	4/4	1/4	
3	Vaccinated	2/3	2/3	0/3	—	0/3	—	—	10,000
	Unvaccinated	—	—	3/3	—	3/3	3/3	3/3	
6	Vaccinated	—	3/3	3/3	2/5	0/5	—	—	1,000
	Unvaccinated	—	—	—	4/4	4/4	4/4	4/4	
8	Vaccinated	—	—	—	5/5	0/4	0/4	—	10
	Unvaccinated	—	—	—	—	4/4	3/4	1/4	
10	Vaccinated	—	—	—	5/5	3/5	0/5	—	10
	Unvaccinated	—	—	—	—	4/4	4/4	4/5	
12	Vaccinated	—	—	—	4/4	3/4	3/4	2/4	±1
	Unvaccinated	—	—	—	—	4/4	4/4	4/4	
20	Vaccinated	—	—	—	—	3/3	3/3	2/3	0
	Unvaccinated	—	—	—	—	—	3/3	3/3	
23	Vaccinated	—	3/3	2/3	3/3	—	3/3	—	0
	Unvaccinated	—	—	—	4/4	4/4	3/4	1/4	

3/3* = three of three injected mice died of encephalitis.
— = dilution not tested.

contrast to that of the resistance tests carried out at this time, which showed the vaccinated mice to be immune to 10,000 M.L.D. of virus injected directly into the brain. Sera collected from mice 2 weeks after vaccination likewise showed little protective action, although

TABLE II

Neutralizing Titre of Sera from Mice Following Vaccination with St. Louis Encephalitis Virus

| Time after vaccination serum obtained | Treatment of mice furnishing serum | Mortality of mice injected intracerebrally with mixture of serum and virus | | | | Degree of protection afforded by serum of vaccinated mice |
| | | Dilution of virus in mixture | | | | Intracerebral M.L.D. |
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	
days						
1	Vaccinated	—	4/4*	3/4	—	<1 (negative)
	Unvaccinated	—	4/4	4/4	0/4	
2	Vaccinated	—	4/4	4/4	1/4	<1 (negative)
	Unvaccinated	—	4/4	4/4	2/4	
wks.						
1	Vaccinated	—	4/4	4/4	—	<1 (negative)
	Unvaccinated	—	4/4	4/4	1/4	
1	Vaccinated	—	4/4	3/4	—	<1 (negative)
	Unvaccinated	—	4/4	3/4	1/4	
2	Vaccinated	—	4/4	2/4	—	<1 (negative)
	Unvaccinated	—	4/4	3/4	1/4	
3	Vaccinated	—	4/4	2/4	—	<1 (negative)
	Unvaccinated	—	4/4	3/4	0/4	
4	Vaccinated	—	4/4	2/4	—	
	"	—	4/4	2/4	—	<1 (negative)
	Unvaccinated	—	4/4	4/4	0/4	
5	Vaccinated	—	4/4	2/4	—	<1 (negative)
	Unvaccinated	—	4/4	4/4	0/4	
5	Vaccinated	—	4/4	1/4	—	±1 (doubtful)
	Unvaccinated	—	4/4	4/4	0/4	
6	Vaccinated	—	4/4	4/4	—	
	"	—	4/4	2/4	—	<1 (negative)
	Unvaccinated	—	4/4	4/4	0/4	
8	Vaccinated	—	2/4	0/4	—	1+ (positive)
	Unvaccinated	—	4/4	4/4	2/4	
8	Vaccinated	—	2/4	0/4	—	1+ (positive)
	Unvaccinated	—	4/4	3/4	1/4	
10	Vaccinated	4/4	1/4	0/4	—	10 (positive)
	Unvaccinated	4/4	4/4	4/4	1/4	
12	Vaccinated	4/4	0/4	0/4	—	10 (positive)
	Unvaccinated	4/4	4/4	4/4	1/4	
12	Vaccinated	—	1/4	1/4	—	10 (positive)
	Unvaccinated	—	4/4	4/4	2/4	
23	Vaccinated	—	1/4	0/4	—	100 (positive)
	Unvaccinated	—	4/4	4/4	4/4	
37	Vaccinated	2/4	0/4	0/4	—	10+ (positive)
	Unvaccinated	4/4	4/4	4/4	1/4	

4/4* = four of four mice injected intracerebrally with serum-virus mixture died of encephalitis.

the vaccinated mice at this time were still able to withstand 10,000 intracerebral M.L.D. of virus. 5 weeks after vaccination two serum pools were tested with doubtful or negative results. At 6 weeks two pools of serum from vaccinated mice were also negative.

The first clearly positive neutralization tests were obtained with two serum pools from mice bled 8 weeks after vaccination. These sera completely neutralized 1 M.L.D. of virus and also protected half the number of the injected mice against 10 M.L.D. At this time the actual immunity of the vaccinated mice was greatly diminished; they
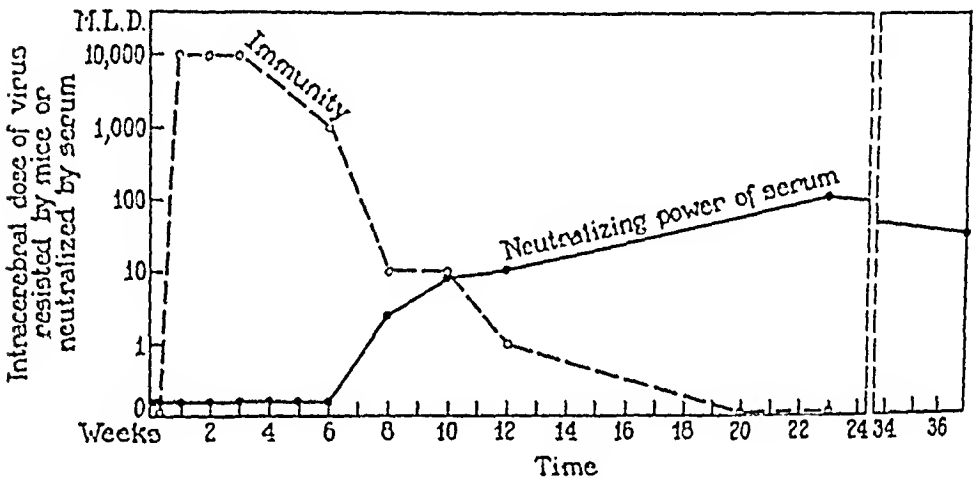
TEXT-FIG. 1. Relation between immunity and neutralizing power of serum following vaccination with St. Louis virus.

were resistant to only 10 intracerebral M.L.D. of virus, whereas they had been immune to 10,000 M.L.D. 3 weeks after vaccination.

At 10 and 12 weeks the sera completely neutralized 1 and 10 M.L.D. of virus. At 23 and 37 weeks the sera completely neutralized 10 M.L.D. and partially inactivated 100 M.L.D. of virus. At this time the actual immunity of the vaccinated mice against intracerebral injection of the virus had entirely disappeared.

The essential point in these comparative titrations of immunity and neutralizing antibodies is the demonstration of variation of these factors in opposite directions over a period of time. When immunity was high, antibodies were undetectable; when immunity was decreasing, antibodies were increasing; and finally, when immunity had

disappeared, antibodies were high (Text-fig. 1). Several experiments other than the one described above have shown the same result.

Experiment 2.—In this experiment a strain of mice inherently resistant to St. Louis encephalitis virus was employed rather than the susceptible animals used in Experiment 1. The resistant mice are refractory to at least 1,000 times the minimal lethal dose for susceptible mice (2). The resistant mice were vaccinated and tested for immunity and for circulating antibodies to determine whether they would produce a more prompt or greater antibody response than that which followed the vaccination of susceptible mice.

The inherently resistant mice showed no neutralizing antibodies before vaccination, indicating that their resistance does not depend chiefly upon a humoral mechanism. 3 weeks after vaccination they

TABLE III

Immunity and Serum Neutralizing Power of Inherently Resistant Mice after Vaccination with St. Louis Encephalitis Virus

Time	Immunity of inherently resistant mice against intracerebrally inoculated virus M.L.D.	Neutralizing power of serum of inherently resistant mice Intracerebral M.L.D.
Before vaccination....................	1,000	0
3 wks. after vaccination................	100,000	0
8 " " "	100,000	1 to 10

withstood the intracerebral injection of 100,000 M.L.D. of virus, but their sera contained no demonstrable neutralizing antibodies. At 8 weeks they resisted 100,000 intracerebral M.L.D. and their pooled sera neutralized 1 M.L.D. completely, and 10 M.L.D. partially.

In brief, inherently resistant mice, following vaccination, develop detectable circulating antibodies no more promptly and in no greater amount than susceptible vaccinated mice.

DISCUSSION

Certain studies on rabies and poliomyelitis indicate that circulating protective antibodies are not necessarily an index of immunity (6–9). These observations have been criticized, however, on the grounds that they are not quantitative, that they refer to a momentary rather than

continuing relationship, and that they may be contradictory under different conditions.

In the present studies with St. Louis virus, however, relatively quantitative data were obtained under one set of conditions and over periods of time during which both the antibody and immunity factors were changing. At the outset we find the brain of the immune animal resistant to 10,000 lethal doses of virus and its serum unable to inactivate one lethal dose. Later, as the acquired immunity falls, antibody titre increases until finally we note animals which have lost their immunity to even one lethal dose but whose circulating antibodies protect against 10 to 100 lethal doses of virus.[2] This variation of immunity and antibody titre in opposite directions is offered as evidence that in St. Louis encephalitis infection in mice, immunity following vaccination does not depend primarily upon the content of circulating neutralizing antibodies.[3]

SUMMARY

Vaccination of susceptible mice with St. Louis encephalitis virus induced an acquired immunity to intracerebral inoculation which appeared within 1 week. When first demonstrable this immunity was at a very high level and it remained so for about 6 weeks, after which time it declined and disappeared completely between the 12th and 20th week after vaccination.

Humoral antibodies of significant titre could not be demonstrated

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[2] Moreover, the inherently resistant mouse which at the outset withstands 1,000 times the dose of virus fatal to the inherently susceptible mouse shows no circulating antibodies before vaccination. They do appear, however, at about the same time following vaccination as they do in the susceptible vaccinated mouse without any apparent relation to the continued high resistance of the animal.

[3] The antibody-immunity relationship is quite different in the case of monkeys vaccinated against poliomyelitis. Such animals may develop neutralizing antibodies without becoming immune to the disease (7-9). However, the antibody response of monkeys convalescent from an attack of poliomyelitis does resemble that which occurs in mice soon after vaccination against the St. Louis virus. In both instances immunity develops before antibodies are demonstrable (9, 10). Whether the immunity induced by an attack of poliomyelitis later disappears while antibodies are still present is not known.

during the first 6 weeks after vaccination when actual immunity to intracerebral inoculation was at its highest level. Antibodies were first detected 8 weeks after vaccination, when immunity was decreasing. The humoral antibody titre reached its peak at 23 weeks, at which time immunity had completely disappeared. A high level of circulating antibody was still present 37 weeks after vaccination.

The evidence presented leads to the conclusion that humoral antibodies do not play a major part in the immunity acquired by susceptible mice after vaccination against St. Louis encephalitis virus.

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SENSITIZATION AND ANTIBODY FORMATION WITH INCREASED RESISTANCE TO TUBERCULOUS INFECTION INDUCED BY HEAT KILLED TUBERCLE BACILLI*

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In a previous publication (1) the immunizing effect of killed tubercle bacilli alone or in combination with other antigens or irritants has been compared with that of an avirulent living tubercle bacillus (BCG) by measuring the resistance of immunized rabbits to infection with highly virulent bovine tubercle bacilli. The evidence presented shows that rabbits immunized with heat killed tubercle bacilli acquire effective protection against infection fatal to unprepared rabbits. A few animals are no more resistant than control rabbits, whereas others with scant lesions live much longer and a considerable number recover from the infection completely. Protection by killed tubercle bacilli affords a new opportunity to study the relationship of sensitization and antibodies to immunity against tuberculosis because the progress of immunization is not modified by a living microorganism which may multiply in the body. One purpose of these experiments has been to determine if heat killed tubercle bacilli can be used to protect human beings against tuberculosis. Hence we have attempted to determine what are the conditions that modify sensitization and antibody production and how these changes affect the local lesion produced by killed tubercle bacilli. Furthermore we have attempted to determine the relation of sensitization and antibody formation to resistance produced by protective inoculation.

Sensitization with Allergic Inflammation of the Skin Induced by Heat Killed Tubercle Bacilli and by BCG and Measured by These Agents or by Tuberculin

The plan of experiment has been to inject a constant amount of heat killed tubercle bacilli into the skin at weekly intervals and observe the skin reactions at the site of injection 2 days later.   These intracutaneous injections have served both to sensitize animals and to measure the progress of sensitization.   The dose, 0.2 mg., has been selected because it is approximately half of that which causes suppuration within 2 days when injected into the skin of normal rabbits.   Injection of 0.2 mg. of heat killed tubercle bacilli produces slight redness and edema in an area about 3 mm. across, raised very little above the skin surface and usually recognizable by touch.   In a few instances the area of edema is 5 mm. across.

The progress of sensitization as shown by allergic inflammation in animals treated with heat killed tubercle bacilli has characters that deserve consideration.   In some animals with successive injections there is a continuous increase in the diameter and height of the inflammatory edema that follows intracutaneous injection, but in others no significant change can be observed after the first 4 or 5 injections.   In most animals local edematous swelling maintains its maximum extent during a period of from 5 to 10 weeks after the beginning of injections, when it usually measures between 30 and 50 mm. in diameter.   Later there is less edema and the lesion at the site of injection is less extensive, but more elevated, reaching occasionally 10 mm. in height.   It is now firmer than in the period of maximum edema, and suppuration, indicated by a yellow spot at the summit of the elevation, often occurs within 48 hours after injection. Suppuration is promptly followed by ulceration and usually after 1 or 2 weeks by complete healing.

The progress of sensitization induced by BCG and measured by the reaction to BCG does not differ essentially from that induced by heat killed tubercle bacilli and tested with heat killed bacilli, but it proceeds in general somewhat more rapidly and edema is more extensive.

With the quantity of old tuberculin that has been used to test sensitization in rabbits, namely, with 0.2 cc. of a 1 in 5 dilution of old tuberculin (40 mg.), allergic inflammation as measured by the diameter of the area of inflammatory edema 48 hours after injection

has been almost identical with that produced by 0.2 mg. BCG. When reactions to this quantity of tuberculin are compared with those produced by 0.2 mg. of heat killed tubercle bacilli, the reactions are in general more extensive with tuberculin.

Variations in the Progress of Sensitization in Different Animals

Rabbits vary widely in the rapidity with which they undergo sensitization following repeated injection with dead tubercle bacilli or with BCG, as indicated by the local inflammatory reaction to the injected substance when introduced into the cutis or to old tuberculin.

TABLE I

Variation in Progress of Sensitization among Rabbits Repeatedly Injected into the Cutis with 0.2 Mg. of Heat Killed Tubercle Bacilli

Diameter of inflam-matory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	Tested with heat killed tubercle bacilli		Tested with tuberculin	
	After 4 to 5 wks.	After 6 to 8 wks.	After 4 to 5 wks.	After 6 to 8 wks.
mm.				
0–9	9	1	4	2
10–19	5	3	2	3
20–29	5	7	3	2
30–29	3	8		3
40–49	1	4	4	2
50–59			1	2
Total.........	23	23	14	14

Table I shows the maximum diameter of redness and edema produced by heat killed tubercle bacilli or by tuberculin after 4 to 5 and after 6 to 8 injections of the former.

Edema less than 10 mm. in diameter may be regarded as evidence of insignificant sensitization and after 4 to 5 weeks 9 of 23 animals have failed to reach this level (Table I). Nevertheless, after 8 weeks only one animal remains below this figure and the general trend in the interval has been conspicuously toward more extensive reactions. Sensitization tested by tuberculin in a smaller number of animals shows similar variation in intensity.

Sensitization of animals repeatedly injected with BCG proceeded more rapidly than that produced by heat killed tubercle bacilli. The trend between 4 and 20 weeks was toward somewhat more severe reactions but differences were inconspicuous.

The foregoing observations show that individual rabbits vary widely in the readiness with which they are sensitized by heat killed tubercle bacilli or by BCG, but few escape conspicuous sensitization after repeated injection of the antigen.

The Fate of Lesions of Allergic Inflammation Produced by Heat Killed Tubercle Bacilli and by BCG

It has been considered desirable to study in rabbits the changes in local lesions produced by heat killed tubercle bacilli because in inoculated persons a single injection of BCG produces skin ulceration and often suppuration of adjacent lymph nodes in a large proportion of instances. The fate of skin lesions produced by the repeated injection of heat killed tubercle bacilli is modified by developing sensitization (Fig. 1).

The lesions, which in general have been produced at intervals of 1 week, have been remeasured each week. The lesion of the first injection is small throughout its course and has disappeared after 5 or 6 weeks before sensitization is well established (see graph at top of Fig. 1). The local lesions of the next 2 or 3 injections persist through a longer period and are still present at a time when sensitization is increasing rapidly. At this time they undergo noteworthy increase in size (see Fig. 1), which is doubtless analogous to the focal increase of tuberculous lesions produced by injection of tuberculin into tuberculous animals. It recalls the enlargement of the local lesions observed by Andrewes, Derick and Swift (2), 8 days after they injected hemolytic streptococci into the skin and coincident with the appearance of sensitization to filtrate from cultures of the microorganism. The progress of sensitization and the corresponding fate of lesions produced by BCG is shown diagrammatically in Fig. 2.

The rapidity with which suppuration and subsequent ulceration occurs at the site of injection into sensitized animals determines the duration of lesions because ulceration in most instances is followed within from 1 to 3 weeks by healing and complete disappearance of the lesion. Lewandowsky (3) showed that ulceration at the site of
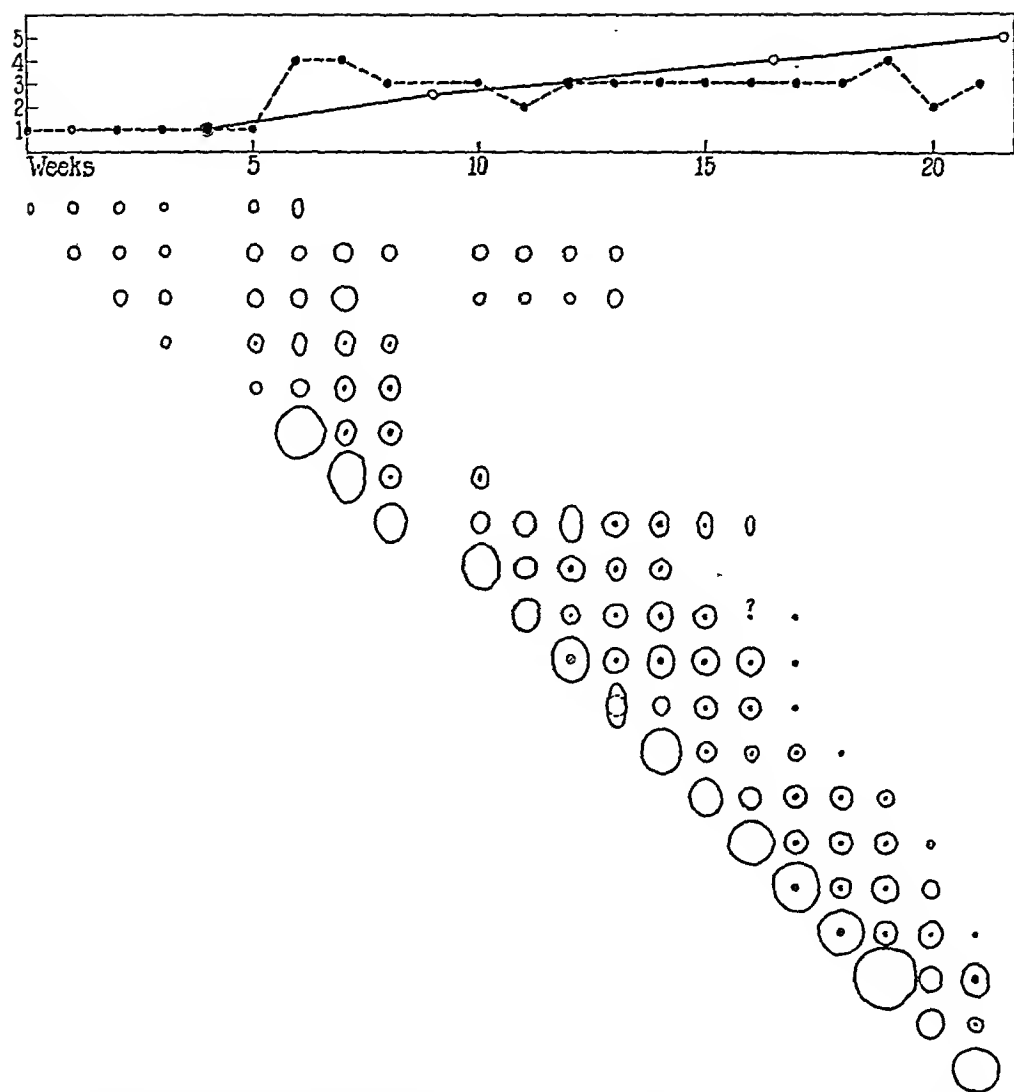
FIG. 1. At the top of the figure is a graph showing the progress of sensitization in a rabbi that received repeated intracutaneous injections of heat killed tubercle bacilli at intervals of week. The solid line represents the relative diameters of skin lesions produced by old tuber culin and the broken line those of lesions produced by heat killed tubercle bacilli. The rela tive size of the lesions produced by weekly intracutaneous injections of tubercle bacilli i shown below the graph and the time at which observations were made is shown by th horizontal scale of weeks, simultaneous observations being in vertical lines below it. In thi and in subsequent graphs the figures at the left of the ordinate indicate sensitizatio measured by the diameter of allergic inflammation, 1 being 8 to 9 mm.; 2, 10 to 19 mm.; ? 20 to 30 mm.; 4, 30 to 40 mm., etc.; or the titer of complement fixation, 1 being 1:5; 2, 1:10; ? 1:20; 4, 1:40; 5, 1:80, etc.
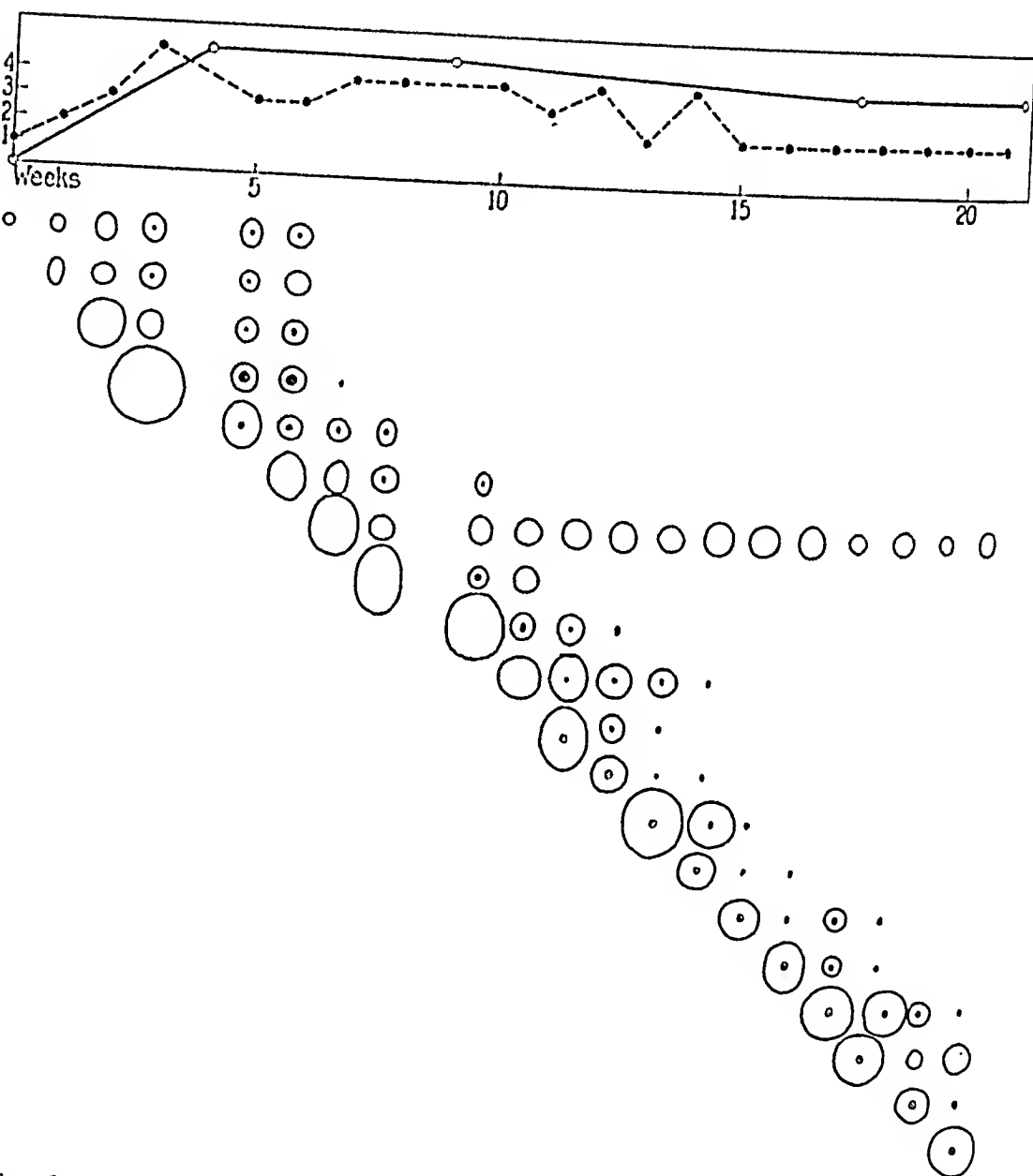
FIG. 2. The progress of sensitization and changes in intracutaneous lesions produced by repeated injections of BCG at intervals of 1 week (for explanation see Fig. 1).   The broken line shows the progress of sensitization as measured by the size of skin lesions produced by BCG.

inoculation of living tubercle bacilli into sensitized animals was associated with the discharge of the microorganism to the exterior in the material that sloughed away. Table II shows the average duration of local lesions produced in 9 rabbits at intervals of 1 week.

When sensitization caused by heat killed tubercle bacilli reaches its maximum lesions persist only about 5 weeks. Intracutaneous lesions following injection of BCG produce suppuration and subsequently heal more promptly than those induced by heat killed

TABLE II

Average Duration of Intracutaneous Lesions in Nine Rabbits Produced at Weekly Intervals by Intracutaneous Injection of 0.2 Mg. of Heat Killed Tubercle Bacilli and of BCG

Time after beginning of injections when lesions were produced	Average duration of intracutaneous lesions produced by heat killed tubercle bacilli	Average duration of intracutaneous lesions produced by BCG
wks.	*wks.*	*wks.*
1	8.2	6.8
2	11.7+	6.5
3	12.0+	6.0
4	8.1+	6.7
5	6.4+	7.1
6	4.7	7.5+
7	9.0+	8.3+
8	9.4+	5.7+
9	6.4	6.5 .
10	4.4	5.7+
11	5.5	3.8
12	5.0	5.1

tubercle bacilli. The persistence of lesions at the site of injection of heat killed tubercle bacilli is a functional index of the persistence of the antigen at the site of its injection. It is improbable that its immunizing activity outlasts its ability to produce a local lesion.

Repeated subcutaneous injection of heat killed tubercle bacilli at intervals of 1 week produces no palpable nodules until sensitization appears. After sensitization small subcutaneous nodules are formed but ulceration rarely occurs.

Sensitization Produced by Intracutaneous and by Subcutaneous Injection of Heat Killed Tubercle Bacilli

Experiments have been undertaken to determine if the progress

of sensitization measured by old tuberculin differs when heat killed tubercle bacilli are introduced into the cutis on the one hand, or into the subcutaneous tissue on the other.

The progress of sensitization following intracutaneous and subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli at intervals of 1 week is shown by composite graphs in Fig. 3, obtained from 9 rabbits injected intracutaneously and 10 injected subcutaneously. Sensitization proceeds more rapidly with the former but after 8 weeks sensitization following subcutaneous injection has reached the same level as that after intracutaneous injection.

In the experiments that follow (Table III), 6 injections of 0.2 mg. of heat killed tubercle bacilli have been given at intervals of 1 week; in the first group of experi-
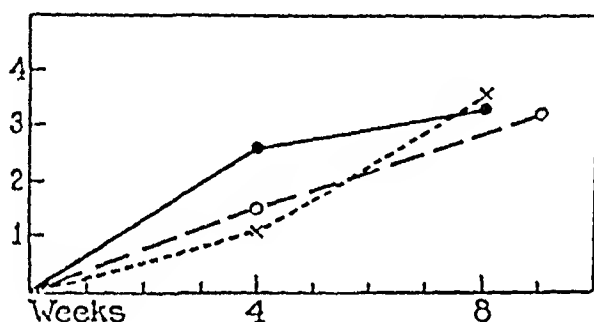
FIG. 3. Composite graph showing the progress of sensitization measured by tuberculin in 9 animals following the intracutaneous (solid line), in 10 animals following subcutaneous (coarsely broken line), or in 13 animals following intravenous (finely broken line) injection of heat killed tubercle bacilli.

ments this quantity has been given each week in a single injection; in the second group it has been divided into 4 simultaneous injections, of which each is 0.05 mg.; and in the third group it has been similarly divided into 8 simultaneous injections, each of 0.025 mg.

The three groups of experiments show uniformly that sensitization proceeds more rapidly following intracutaneous than following subcutaneous injection, so that after 4 weekly injections a smaller number of animals resist sensitization and in general reactions are more intense. After 8 weekly injections reactions are stronger and all animals have become well sensitized.

Effect of Different Quantities of Heat Killed Tubercle Bacilli upon the Progress of Sensitization

In order to determine the effect of a minimal stimulus upon the production of sensitization a single injection of 0.2 mg. of heat killed

tubercle bacilli has been injected into the subcutaneous tissue and the progress of sensitization compared with that produced by 4 injections of the same quantity of heat killed tubercle bacilli adminis-

TABLE III

Progress of Sensitization to Tuberculin with Cutaneous and Subcutaneous Injection of Heat Killed Tubercle Bacilli and with Undivided and Divided Doses

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	With intracutaneous injection		With subcutaneous injection	
	During 4 wks.	During 8 wks.	During 4 wks.	During 8 wks.
mm.				
With injections of 0.2 mg. each week				
0–9	1		2	
10–19				
20–29	1		2	
30–39		1		1
40–49	3	2	1	1
50–59		2		2
60–69				1
With 4 simultaneous injections of 0.05 mg. each week				
0–9			2	
10–19	1		1	
20–29				
30–39	1	1	2	
40–49	2	2		4
50–59		1		1
With 8 simultaneous injections of 0.025 mg. each week				
0–9			2	
10–19			1	
20–29	2			
30–39	2	1		1
40–49		2	1	1
50–59	1	1	1	3
60–69		1		

tered at intervals of 1 week (Table IV; the progress of antibody formation in the same experiment is shown in Table VIII).

The experiment shows that 0.2 mg. of heat killed tubercle bacilli in 1 injection is ineffective in the production of sensitization in rabbits and even after 4 injections at intervals of 1 week few animals have become sensitized. Sensitization with BCG in 1 or in 4 injections

has been much more successful. Nevertheless, after an interval of rest, that is, after 6 weeks (Table IV), these differences have in considerable part disappeared.

Injections of 0.2 mg. of heat killed tubercle bacilli continued during 6 weeks have been much more effective in the production of sensitization that 1 or 4 similar injections (Table IV). In one experiment increasing the initial dose from 0.2 mg. of heat killed tubercle bacilli (10 animals) to 7 times this amount (39 animals) has had little effect upon the progress of sensitization judged by the number that resist sensitization (with lesions less than 10 mm. in diameter) after

TABLE IV

Progress of Sensitization to Tuberculin Produced by a Single Injection of Heat Killed Tubercle Bacilli or of BCG Compared with That Following Four Similar Injections at Intervals of 1 Week

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left											
	Heat killed tubercle bacilli						BCG					
	With 1 injection			With 4 injections			With 1 injection			With 4 injections		
	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.
mm.												
0–9	9	6	7	9	6	7	4	2	5	3		5
10–19		2	2		1		2	1	1	2		2
20–29		1			1		1		1	1	1	1
30–39					1	1	2	5	1	2	5	1
40–49								1		2	2	1
50–59											2	

4 weeks, but has increased the proportion of those with stronger reactions (30 to 49 mm. in diameter). With an increase of the injected dose from 0.2 to 0.4 mg. (24 animals) there has been uniform diminution in the proportion of rabbits that resist sensitization and an increase of the proportion with strong reactions.

Sensitization Following Intravenous Injection of Heat Killed Tubercle Bacilli

Intravenous injection of dead tubercle bacilli has proven to be a relatively ineffective and very dangerous method of producing sen-

sitization. The progress of sensitization following intravenous, intracutaneous and subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli at intervals of 1 week is shown by the composite graphs in Fig. 1, of which that showing sensitization induced by intravenous injection has been obtained from 13 animals. It is evident that sensitization following intravenous and subcutaneous injection of heat killed tubercle bacilli proceeds more slowly than that after intracutaneous injection, so that after 4 weeks sensitization with intracutaneous injection is much stronger. After 8 weeks, sensitization following both subcutaneous and intravenous injection has increased and reached the level of that induced by intracutaneous injections. There has been no essential difference in the progress of intravenous and subcutaneous sensitization.

TABLE V

Progress of Sensitization after Intravenous Weekly Injections of 0.2 Mg. of Heat Killed Tubercle Bacilli

Diameter of inflammatory reaction	After 4 wks.	After 8 wks.
mm.		
0–9	8	5
10–19	1	
20–29	2	2
30–39	1	2
40–49	1	
50–59		3

The range of reactions is better shown by Table V, in which it is evident that a considerable number of animals resist sensitization though a few become well sensitized.

Intravenous injection of heat killed tubercle bacilli into animals that are highly sensitized is attended by considerable risk. 2 of the rabbits that have developed intense sensitization, included in Table V, have died within 1 day, and 2 after 1 week, following the intravenous injection of heated tubercle bacilli.

Antibody Formation

Antibody formation has been studied in the animals that have been used to follow the progress of sensitization and the plan of the experiments has been the same.

The complement fixation tests have been performed with sera heated for 30 minutes at 55°C. and a suspension of the Ravenel strain has been used as antigen. The latter is prepared by suspending a culture with the aid of grinding in normal saline so that 1 cc. of the suspension contains 10 mg. of tubercle bacilli. The suspension is heated for 30 minutes at 60°C. and 0.35 per cent tricresol is added as a preservative. About one-fourth of the self-inhibition dose is used for the complement fixation. The three ingredients, namely, the serum to be tested, the antigen suspension and the guinea pig serum containing complement, are mixed and incubated in a water bath at 37°C. for 1 hour. Then rabbit serum containing 2 units of hemolysin and a 5 per cent suspension of sheep blood cells are added to the mixture and incubated in the water bath until the control serum indicates the completion of the reaction. To check the accuracy of the complement fixation tests, a standard serum which is obtained from a rabbit immunized by a series of

TABLE VI

Variation in Progress of Antibody Production among Rabbits after Six Injections of Heat Killed Tubercle Bacilli

Titer	Number of rabbits with antibody titers indicated in column at left					
	With intracutaneous injection			With subcutaneous injection		
	After 4 wks.	After 6 wks.	After 10 wks.	After 4 wks.	After 6 wks.	After 10 wks.
0	2	1		4	1	
5	2		2	1		2
10		1	1		2	1
20		2	2		2	2
40	1					
80		1				

injections of heat killed tubercle bacilli is included in each test. Measured quantities (0.5 cc.) of the serum are dried *in vacuo* in the frozen state by the method of Elser, Thomas and Steffen (4), and a sample of the serum, recovered by adding 0.5 cc. of distilled water to the dry material, is tested for complement fixation. Tests made simultaneously with three samples of dried serum show that the method of drying yields uniform samples.

Individual Variation of Antibody Production in Rabbits after Repeated Injections of Heat Killed Tubercle Bacilli

One group of 5 animals has received into the skin weekly injections of 0.2 mg. heat killed tubercle bacilli for 6 weeks and another group has received the same injections into the subcutaneous tissue. Antibody titers are determined by complement fixation test 5 days after

the fourth injection, 5 days after the sixth injection, and approximately 10 weeks after the beginning of the experiment. The results are shown in Table VI.

The titers of the sera that have fixed complement vary widely in different rabbits. Individual variation of the same order is present in the group with both intracutaneous and subcutaneous injections.

Progress of Antibody Formation after Intracutaneous, Subcutaneous and Intravenous Injection of Heat Killed Tubercle Bacilli

Parallel experiments with varied methods of administration of heat killed tubercle bacilli have been made, on the one hand, with

TABLE VII

Progress of Antibody Formation after Subcutaneous and after Intravenous Injection of Heat Killed Tubercle Bacilli

Titer	Number of rabbits with antibody titers indicated in column at left					
	With weekly injections of 0.2 mg.					
	With subcutaneous injection			With intravenous injection		
	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.
0						
5	1					
10	2	2				
20	1	3	2			
40	1		2	1		
80			1	1		
160				1	1	
320					1	2
640					1	1

intracutaneous, and on the other, with subcutaneous, injection. Antibody formation has proceeded somewhat more rapidly with intracutaneous than with subcutaneous injections but the difference is not conspicuous.

Antibody titers of the blood rise faster and reach higher levels after weekly intravenous than after weekly subcutaneous inoculations of 0.2 mg. of heat killed tubercle bacilli (Table VII). In another group of seven rabbits injected intravenously at intervals of 1 week with amounts of heat killed tubercle bacilli ranging from 0.003 to

0.05 mg. the antibody titers have been lower than in animals that have received weekly intravenous injections of 0.2 mg. of vaccine, but higher than in those treated with weekly subcutaneous injections of 0.2 mg. heat killed tubercle bacilli.

Antibody Formation with Multiple Simultaneous Injections

Multiple simultaneous intracutaneous or subcutaneous injections of heat killed tubercle bacilli produce antibodies in greater titer than the same quantity in a single injection. Of 10 animals that received a single injection of 0.2 mg. each week (see Table III), only one had

TABLE VIII

Progress of Antibody Formation after One Subcutaneous Injection or after Four Weekly Injections of Heat Killed Tubercle Bacilli and of BCG

Titer	Number of rabbits with antibody titers indicated in column at left							
	Heat killed tubercle bacilli				BCG			
	At 2 wks.	At 4 wks.	At 6 wks.	At 8 wks.	At 2 wks.	At 4 wks.	At 6 wks.	At 8 wks.
	After 1 subcutaneous injection of 0.2 mg.							
0	9	8	9	9	9	6	3	4
5		1				3	3	1
10							2	2
20								1
	After 4 weekly subcutaneous injections of 0.2 mg.							
0	9	9	7	7	9	1	2	2
5			1	1	1	4	4	5
10						2	3	3
20						3	1	

antibodies in a titer of 40 or more after 6 weeks; of 9 animals that received 4 injections of 0.05 mg. each week, 5 had titers of 40 or more, and of 10 animals that received 8 injections of 0.025 mg. each week, 6 had titers of 40 or more.

Progress of Antibody Formation after Injection of Heat Killed Tubercle Bacilli or of BCG

When rabbits have received one subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli or the same quantity 4 times at intervals of 1 week, antibody formation has been scarcely demonstrable

(Table VIII; the progress of sensitization in the same experiment is shown in Table IV), but when BCG has been administered by the same procedure antibody formation has been observed in most of the rabbits.

In experiments with 4 simultaneous injections of 0.1 mg. repeated at weekly intervals for 4 weeks (Table IX), the antibody titers in the group treated with BCG are slightly higher after 4 weeks than in those injected with heat killed tubercle bacilli, but the relationship is reversed at the end of 6 and 8 weeks.

The experiment shows that small quantities of BCG have induced much more abundant antibody formation than heat killed tubercle

TABLE IX

Progress of Antibody Formation after Four Simultaneous Injections (0.1 Mg.) of Heat Killed Tubercle Bacilli or of BCG at Intervals of a Week for 4 Weeks

Titer	Number of rabbits with antibody titers indicated in column at left							
	Heat killed tubercle bacilli				BCG			
	After 2 wks.	After 4 wks.	After 6 wks.	After 8 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 8 wks.
0	8	3	1	1	17	2	4	7
5	1	2	2	3	1	5	2	3
10		2	3	2		3	6	5
20		1	1	3		5	4	3
40		1	2			2	2	
80						1		

bacilli, but when the amount of vaccine has been increased the two kinds of vaccine have been almost equally effective in producing antibodies measured by complement fixation.

The Relation of Sensitization and Titer of Antibody (Complement Fixation) Preceding Infection to Resistance against Infection

Animals repeatedly inoculated with heat killed tubercle bacilli or with BCG become, on the one hand, sensitized as indicated by the cutaneous tuberculin reaction and form antibodies actively as measured by complement fixation, and on the other hand acquire increased resistance to infection as revealed by complete recovery from

infection, prolonged survival after infection, or diminished extent of tuberculous lesions. Animals that exhibit increased resistance are sensitive to products of the tubercle bacillus and have humoral antibodies at the time when they are infected, but no exact parallel has been found between either the intensity of sensitization or the titer of antibody and resistance. It is doubtful if any parallel should be expected even though sensitization, antibody formation and resistance are dependent upon a common factor, for each may be dependent upon other variable factors which cannot be kept constant.

Sensitization or allergic inflammation measured by the maximum intensity of the tuberculin reaction before infection may be compared with the extent of pulmonary lesions, the duration of life after infection, or complete recovery from infection. Gradations in resistance to infection may be roughly defined: (a) The least resistant of the "vaccinated" animals are those that die within 300 days and at autopsy, like most of the infected controls, are found to have one-half or more of the lungs consolidated by tuberculous lesions. (b) Animals that die within 300 days and have less than half of the lungs involved have evidently shown greater resistance, not exactly measurable by the length of survival after infection because intercurrent disease may have hastened their end. (c) Another group of animals, in general more resistant than the foregoing, are those that survive more than 300 days. (d) The most resistant animals are those that have had no tuberculosis discoverable after death. Control animals after infection with 0.00001 mg. of virulent bovine tubercle bacilli have died in all instances and with few exceptions within 200 days after infection; from four- to nine-tenths of the cut section of the lung substance is usually occupied by tuberculous tissue. Table X shows the fate of animals with varying intensity of sensitization preceding inoculation with virulent tubercle bacilli.

A few animals of both the susceptible and the resistant groups (Table X), on the one hand, have exhibited scant sensitization before infection (reactions with diameter from 0 to 9 mm.). On the other hand, a small number of animals that have become intensely sensitized (reactions with diameter from 50 to 69 mm.) have exhibited minimum resistance to infection.

The attempt has been made to determine if there is any relation

between the titer of complement fixation of immunized animals before infection and their subsequent resistance to the disease (Table XI).

There is evidently no relation between the titer of complement fixation before infection and resistance of the animal so constant that it may be used to predict the fate of the animal.

TABLE X

Relation of Sensitization before Infection, as Indicated by the Maximum Tuberculin Reaction, to Resistance

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	Surviving infection less than 300 days and with half of lung involved by tuberculous lesions	Surviving infection less than 300 days and with less than half of the lung involved	Surviving from 300 to 600 days	Living after 700 days
mm.				
0–9	2		3	2
10–19	3		3	2
20–29	2	4	13	3
30–39	5	20	20	10
40–49	6	7	8	9
50–59	3	1		
60–69	2		1	1
Total number of animals.......	23	32	48	27

Sensitization and Complement Fixation during the Course of Tuberculous Infection and Their Relation to Resistance

Sensitization or complement fixation before infection is doubtless a poor index of its influence upon the latter because during the course of long continued disease there is abundant opportunity for alteration of preexisting relations. To measure the influence of sensitization, as determined by intracutaneous tuberculin tests, and of antibody formation, determined by complement fixation with blood serum, upon the progress of infection, it is desirable to compare: (*a*) sensitization and complement fixation during the course of infection with no preceding immunization (infected controls); (*b*) changes in

sensitization and complement fixation in immunized animals that have not been infected; (c) sensitization and complement fixation in immunized animals that have been infected.

Changes in sensitization and antibody formation during the progress of tuberculous infection in rabbits have been studied by Freund, Laidlaw and Mansfield (5). In animals that have received 0.00001 mg. of bovine tubercle bacilli intravenously, sensitization becomes evident from 2 to 6 weeks after infection, increases rapidly and then fluctuates somewhat in intensity. During a period that has usually

TABLE XI

Relation of Complement Fixation before Infection to Resistance

	Number of rabbits with maximum complement fixation as indicated in column at left			
Titer of complement fixation	Surviving infection less than 300 days and with half of lungs involved by tuberculous lesions	Surviving infection less than 300 days and with less than half of the lungs involved	Surviving from 300 to 600 days	Living after 700 days
0				
5				
10	1			
20		2	1	2
40	3	7		
80	5	5	3	4
160		3		
Total number of animals......	9	17	4	6

varied from 2 to 8 weeks before death, the animals have failed to react to tuberculin. Complement-fixing antibodies have appeared in the blood within 2 weeks after infection, increased during 6 to 10 weeks, and subsequently maintained a fairly constant level, remaining elevated during the terminal period in which sensitization has disappeared. Fig. 4 is a composite graph showing the course of sensitization and complement fixation in animals infected intravenously with 0.00001 mg. of bovine tubercle bacilli. It is noteworthy that a few animals infected with this dose form no antibodies demonstrable by complement fixation.

The changes in sensitization and complement fixation following immunization produced by repeated subcutaneous injection of heat killed tubercle bacilli during a period of 8 to 12 weeks is shown in Fig. 5, which is a composite graph recording observations on 17 animals. 4 weeks after the beginning of immunization both sensitiza-
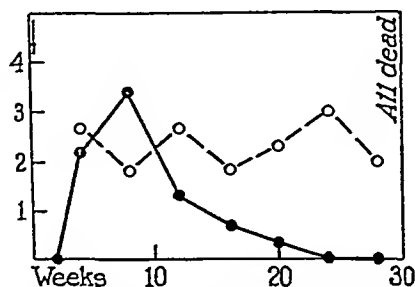
FIG. 4. Composite graph showing the progress of sensitization measured by tuberculin (solid line) and of antibody formation determined by complement fixation (broken line) in 9 animals that were intravenously infected with 0.00001 mg. of bovine tubercle bacilli.
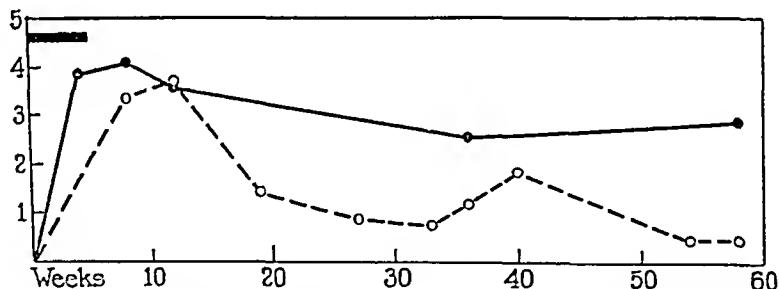
FIG. 5. Composite graph showing the progress of sensitization (solid line) and of antibody formation determined by complement fixation in 17 animals that received intracutaneously 8 to 12 injections of heat killed tubercle bacilli at intervals of 1 week.

tion and complement fixation have reached a high level. With no immunizing injections after the 12th week, tuberculin sensitization remains elevated with only slight diminution for at least 1 year but complement fixation, which has reached a maximum about 12 weeks after the beginning of the injection of heat killed tubercle bacilli, gradually falls and in most animals (12 of the 17 included in the

graph) disappears for a time completely. An almost constant feature of these curves is the subsequent rise of complement fixation. It has occurred between the 30th and 40th weeks and has been sharply defined in 14 of the 17 animals included in Fig. 5, and is present, though ill defined, in the remaining 3.

In animals infected after immunization with heat killed tubercle bacilli, the resistance of the animal is indicated by the duration of life greater or less than 300 days, and by the extent of tuberculosis found after death, measured roughly by the proportion of lung occupied by tuberculous tissue, in accordance with the groups defined above (e.g. in Tables X and XI). In an animal that has died within 300 days after infection with half or more of the lung replaced by tuberculous lesions there has evidently been little resistance to the disease (Fig. 6, graph 1), and it is noteworthy that sensitization and antibody formation demonstrable by complement fixation have followed the same course as that of the infected control (Fig. 4), sensitization disappearing several weeks before death although complement fixation remains elevated. In an animal that has shown more resistance to infection so that within 300 days after infection only one-tenth of the lung is occupied by tuberculous tissue (Fig. 6, graph 2), the course of sensitization has been at first approximately the same as that of the infected control and of the immunized animal that has exhibited no resistance. Following infection sensitization measured by the tuberculin test falls rapidly and after from 20 to 30 weeks has for a time completely disappeared, but complement fixation, as in the infected control, remains at a high level. In the least resistant of the immunized animals as in the control, sensitization fails to reappear, but in the more resistant animals with longer period of survival and less extensive tuberculosis sensitization reappears but disappears again before death occurs.

In animals that have shown maximum resistance, indicated by complete recovery from infection, the early course of sensitization and antibody formation (Fig. 6, graph 3) has differed little from that of the control infected animal or of the immunized animal that has died with tuberculosis; sensitization has disappeared after 30 weeks but complement fixation has remained elevated. Sensitization again increases and then falls to zero; this rise to a considerable
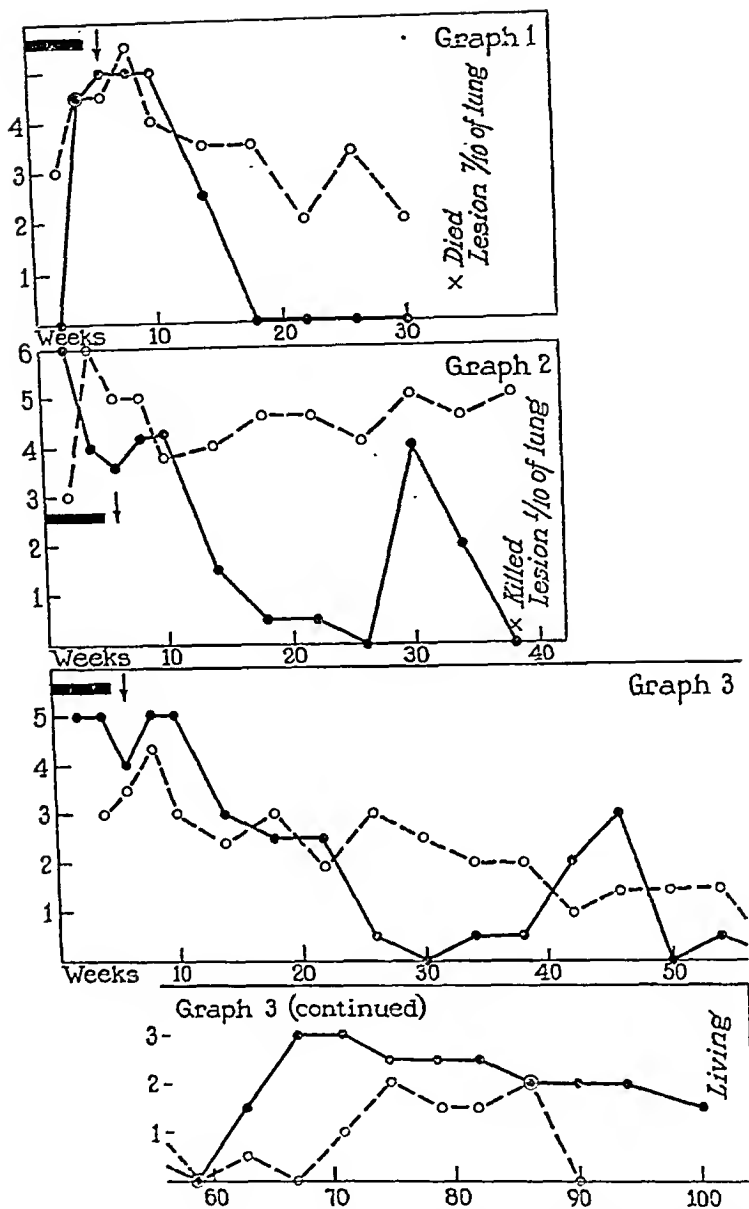
Fig. 6. Graph 1 shows the progress of sensitization (solid line) and of antibody formation (broken line) in a rabbit that received six intracutaneous injections of heat killed tubercle bacilli and showed scant resistance to intravenous infection with bovine tubercle bacilli, dying after 30 weeks with seven-tenths of the lung substance occupied by tuberculous lesions. Graph 2 shows the progress of sensitization and antibody formation in an animal that showed moderate resistance to infection, dying after 40 weeks with only a tenth of the lung occupied by tuberculous lesions. Graph 3 shows the same in an animal with such complete resistance that no tuberculosis was found when the animal was killed 111 weeks after infection.

293

height, followed by complete disappearance, may occur repeatedly (Fig. 6, graph 3). Complement fixation fluctuates less conspicuously and its course does not coincide with that of sensitization.
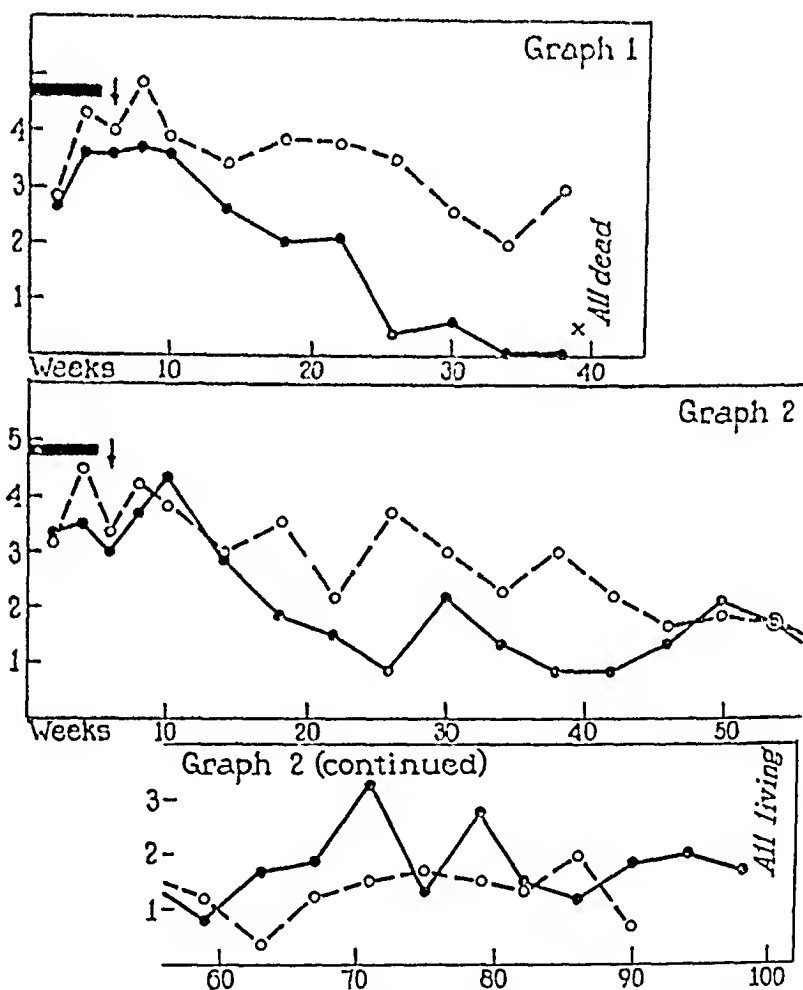
FIG. 7. Graph 1 is a composite showing the progress of sensitization and antibody formation in 9 "vaccinated" animals that showed scant resistance to tuberculous infection (see Fig. 6, graph 1). Graph 2 is a composite showing the progress of sensitization and antibody formation in 6 "vaccinated" animals that resisted tuberculous infection (see Fig. 6, graph 3).

It tends to diminish gradually in animals that recover from infection. Its course resembles that following immunization with no infection but the fall is more gradual and more prolonged.

The trend of sensitization and of complement fixation is well shown in Fig. 7, in which graph 1 is a composite of 9 immunized animals that died within 300 days after infection with more than half of the lungs occupied by tuberculous lesions. It resembles closely those from which it is formed (see Fig. 6, graph 3). The composite graph (Fig. 7, graph 2) of animals that have been most resistant to infection, surviving more than 700 days, does not show the usual course of sensitization as well as the selected graph (Fig. 6, graph 3), because in 5 of the 6 animals from which it is prepared sensitization has repeatedly fallen to the base line and then reappeared, whereas it has remained continuously elevated in only one animal. The titer of complement fixation is more constantly elevated but has gradually and almost continuously decreased.

<center>DISCUSSION</center>

It has long been recognized that resistance to tuberculosis may occur in the absence of sensitization revealed by allergic inflammation; the dog and rat which unlike the guinea pig and the rabbit acquire no skin sensitization to tuberculin when they are infected with tubercle bacilli are highly resistant to the disease. Examples of specific acquired immunity in the absence of sensitization or "allergy" might be multiplied readily. It is more important to know in what degree and within what limitations sensitization or allergic inflammation (the word is here used in the sense defined by von Pirquet (6), who introduced it) when it occurs, increases or decreases resistance against tuberculous infection.

When rabbits are immunized by BCG under varied conditions, some, Clawson (7) states, react to tuberculin and others acquire no demonstrable sensitization; nevertheless, both groups, he says, show some resistance against infection, indicated by the extent of tuberculous lesions in animals killed 3 months after inoculation. It is noteworthy that these experiments in which sensitization has been measured before infection give no decisive information concerning its relation to resistance because during the long course of infection there is abundant opportunity for fluctuation in its intensity. Our experiments in which rabbits were given from 1 to 6 or more injections of heat killed tubercle bacilli (Tables I and IV) show that the ap-

pearance of sensitization may be much delayed and fail to appear after several injections. Successive injections seem to have a cumulative effect so that when they are continued sensitization finally ensues and similar increase of sensitization may follow infection of a partially sensitized animal.

The graphs of sensitization and antibody formation during the course of tuberculous infection show unexplained fluctuations. Changes of sensitization and of antibodies are not parallel. The intensity of sensitization diminishes and disappears with advancing disease and with fatal infection sensitization is usually absent during a considerable period before death, though antibodies indicated by complement fixation persist. Also with tuberculosis in immunized animals that pursues a favorable course, sensitization usually disappears but may reappear in waves throughout the course of recovery. When the animal loses its capacity to react with restored sensitization, death occurs. The disappearance of sensitization in infected animals preceding death has not been satisfactorily explained; the fluctuations in its intensity with its periodic disappearance, observed in immunized and infected animals, are doubtless of similar character. It is probable that desensitization by excess of antigen formed during the progress of infection has a part in this phenomenon.

These relations of antigen to antibodies, and of sensitization to resistance, suggest that we are measuring antibodies or other factors that are formed in excess of the quantity needed to combat infection. Those that are actually concerned in agglutination or phagocytosis or other antibacterial activity are perhaps absorbed by the tubercle bacillus and are no longer demonstrable in the serum or measurable by skin tests. With the progress of recovery stimulation of antibody formation diminishes and the measurable excess in the serum falls.

SUMMARY AND CONCLUSIONS

Rabbits (and human beings) differ widely in the rapidity with which they undergo sensitization with heat killed tubercle bacilli, but after repeated injections all animals become sensitized.

Intracutaneous injection of a small quantity of heat killed tubercle bacilli into a previously normal animal produces a nodule which persists from 8 to 12 weeks; the same injection into well sensitized

animals produces a lesion which ulcerates within from 1 to 3 weeks and is completely healed after about 5 weeks. Complete healing is functional evidence of the disappearance of the antigen.

Intracutaneous injection of heat killed tubercle bacilli induces more rapid sensitization than subcutaneous or intravenous injection, but after repeated injections the difference disappears.

Increasing quantities of heat killed tubercle bacilli or the same quantity divided into several simultaneous injections accelerates sensitization.

The rapidity of antibody formation measured by complement fixation varies in different rabbits under the same conditions but complement fixation is always demonstrable after repeated injections of heat killed tubercle bacilli. Antibody formation is more rapid and reaches higher titers with intravenous than with intracutaneous or with subcutaneous injections. It is accelerated by division of the injected antigen into multiple simultaneous injections.

Small quantities of BCG induce rapid sensitization and more abundant antibody formation measured by complement fixation than heat killed tubercle bacilli but with repeated injections the difference disappears.

Animals that are sensitized and immunized (allergic) before infection are in most instances more resistant to infection than previously normal animals, but there is no correlation between the intensity of sensitization or the titer of antibodies, on the one hand, and resistance to infection on the other.

A previously normal animal subjected to infection differs essentially from a sensitized and immunized animal during the first few weeks of infection when sensitization and immunity are developing as the result of infection, but subsequently the progress of sensitization and antibody formation measured by the means at our disposal follows for a time the same course in both. Sensitization diminishes and in most instances disappears, whereas the titer of complement fixation remains elevated.

When infection pursues a fatal course sensitization permanently disappears, but in animals that proceed toward recovery sensitization measured by injection of tuberculin into the skin repeatedly diminishes, usually to complete disappearance, and then increases in suc-

cessive waves which tend to diminish in height with recovery from infection.

The titer of complement fixation gradually diminishes with recovery from infection.

It is probable that the skin test for sensitization and complement fixation applied to the blood serum measure antibodies or other factors determining sensitization and immunity that are in excess of those actively concerned in the maintenance of resistance.

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THE EFFECT OF SEX HORMONES ON THE RENAL EXCRETION OF ELECTROLYTES*

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In earlier studies (1, 2) it was observed that subcutaneous injections of suprarenal cortical extract were accompanied by a retention of sodium and chloride, and an increased renal excretion of potassium in normal human subjects and normal dogs as well as in suprarenalectomized dogs and patients suffering from Addison's disease. A similar effect was observed in normal dogs (3), following the injection of corticosterone and 11-dehydro-corticosterone (crystalline compounds derived from the suprarenal cortex) and desoxy-corticosterone acetate (synthesized by Steiger and Reichstein (4)). All three of these substances have been shown to be effective in maintaining suprarenalectomized dogs. The discovery that a close structural relationship exists between the compounds with cortin-like activity and the sex hormones (5) (particularly progesterone) as well as the observation that estrus, pseudopregnancy and pregnancy prolong the survival of suprarenalectomized animals (6–8) prompted a study of the effect of injections of sex hormones on the renal excretion of electrolytes in normal and suprarenalectomized dogs. In a preliminary report (9) the sodium retaining effect of the sex hormones has been described.

Methods

Male dogs (approximately 10 kilos) were maintained in metabolism cages and fed a constant diet. The care of the animals, the preparation of the diet and the

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technique used in the metabolism studies have been described previously (2, 3). At the completion of each 24 hour period the animals were catheterized and the total 24 hour urine specimens were collected, preserved with toluene and analyzed. The substances under investigation were injected subcutaneously in either mazola or sesame oil, the possible effect of the solvent being controlled by suitable experiments.

In the normal dog the subcutaneous injection of suprarenal cortical extract (240 to 400 gm. of fresh cortex), corticosterone (4 mg.), 11-dehydro-corticosterone (4 mg.) and desoxy-corticosterone acetate (1 mg.) resulted in a marked decrease in the renal excretion of sodium and chloride and an increased renal excretion of potassium during the 24 hour period in which the injections were made. During the second 24 hour period a rebound effect occurred which was characterized by an increased excretion of sodium and chloride and a decreased excretion of potassium. Frequently the additional loss of sodium and chloride which occurred during the second 24 hour period was equal to the amount retained during the previous 24 hours (3). It is noteworthy that this effect always occurred during the second 24 hour period. Neither the quantity of material injected nor the solvent (aqueous or oil solution) appeared to alter the time relationship of this response.

Two inactive crystalline compounds, (allopregnane-3, 11, 17, 20, 21-pentol and allopregnane-3, 17, 20-triol) derived from the suprarenal cortex failed to give this response in quantities as great as 10 mg.

Progesterone

The effect of progesterone[1] was studied in six normal male dogs and in one suprarenalectomized male dog. In the normal dogs a

Under spinal anesthesia a bilateral suprarenalectomy was performed by Dr.

single subcutaneous injection of 1 to 5 mg. of progesterone in 1 cc. of either mazola or sesame oil had no appreciable effect on the 24 hour renal excretion of electrolytes. A single subcutaneous injection of 15 to 20 mg. of progesterone in 3 to 4 cc. of either mazola or sesame oil resulted in a decreased renal excretion of sodium and chloride during the 24 hour period immediately following the injection (Table I). A slight increase in the renal excretion of potassium usually occurred during this period. The effect of progesterone appeared to reach its maximum during the 24 hour period immediately following injection. In the second 24 hour period the renal excretion of sodium

TABLE I

The Effect of Progesterone on Renal Excretion in a Normal Male Dog (1)*

24 hour period	Body weight	Urine volume	Sodium	Chloride	Potassium	Inorganic phosphorus	Total nitrogen	Treatment
	kg.	*cc.*	*m.eq.*	*m.eq.*	*m.eq.*	*gm.*	*gm.*	
May 16–17	13.35	530	56.8	56.5	18.7	0.41	9.5	Control
" 17–18	13.40	400	42.8	42.1	19.4	0.46	9.7	Progesterone†
" 18–19	13.40	625	56.1	54.8	16.2	0.45	9.6	Control

* Throughout this study the animal was maintained on a constant diet which contained 62 m.eq. of sodium, 63 m.eq. of chloride and 30 m.eq. of potassium per day.

† A single subcutaneous injection of 20 mg. of progesterone in 4 cc. of sesame oil.

and chloride approached normal values. No rebound effect was observed in these experiments. In this respect the effect of progesterone differed from the effect of corticosterone, 11-dehydro-corticosterone and desoxy-corticosterone acetate. Like the naturally occurring and synthetic cortin-like substances, progesterone (20 mg.)

had no appreciable effect on the renal excretion of inorganic phosphorus and total nitrogen.

The withdrawal of suprarenal cortical extract in the treatment of suprarenalectomized dogs is followed by a prompt diuresis, an increased renal excretion of sodium and chloride and a decrease in body weight. This effect reaches its maximum within 24 hours (Table II). A single injection of 20 mg. of progesterone in 4 cc. of mazola oil delayed the onset of the sodium and chloride diuresis and

TABLE II

The Effectiveness of Progesterone in Delaying the Sodium Diuresis Which Follows the Withdrawal of Suprarenal Cortical Extract Treatment in a Suprarenalectomized Dog (2)*

24 hour period	Body weight	Urine volume	Sodium	Treatment
	kg.	cc.	m.eq.	
1937	11.70	600	63.1	Suprarenal cortical extract†
Dec. 7–8	11.80	590	64.1	Progesterone‡
" 8–9	11.55	740	100.8	No treatment
" 9–10			Control Experiment	
1938	14.60	460	64.2	Suprarenal cortical extract†
Jan. 14–15	14.35	780	126.4	No extract
" 15–16	14.20	780	111.5	" "
" 16–17				

* The animal was maintained on a constant diet which contained 62 m.eq. of sodium per day.

† Suprarenal cortical extract 4 cc. (160 gm. suprarenal cortex) injected subcutaneously at 9 a.m. and 4 p.m.

‡ Progesterone (20 mg. in 4 cc. of mazola oil), as a single subcutaneous injection at 9 a.m.

weight loss in a suprarenalectomized male dog after treatment by suprarenal cortical extract had been discontinued (Table II). The duration of the response was similar to that observed in normal dogs, i.e. 24 hours. The large quantities of progesterone necessary (20 mg. or more per day) precluded more extended observations on suprarenalectomized dogs.

Estrone and α-Estradiol

The effect of estrone and α-estradiol was observed in five normal male dogs, two suprarenalectomized male dogs and one female patient

with Addison's disease. In the normal dogs a single subcutaneous injection of α-estradiol (5 mg.), estrone (15 mg.) or amniotin (40,000 to 100,000 International units) resulted in a marked decrease in the renal excretion of water, sodium, chloride, inorganic phosphorus and total nitrogen, potassium excretion being slightly increased (Table III). The duration of this effect varied between 24 and 72 hours, the effect of amniotin and α-estradiol (48 to 72 hours) being somewhat more prolonged than that of crystalline estrone (24 hours).

TABLE III

The Effect of α-Estradiol on the Renal Excretion of Electrolytes in a Normal Male Dog (3)*

24 hour period	Body weight	Urine volume	Sodium	Chloride	Potassium	Inorganic phosphorus	Total nitrogen	Treatment
1937	*kg.*	*cc.*	*m.eq.*	*m.eq.*	*m.eq.*	*gm.*	*gm.*	
Mar. 29–30	11.25	425	55.2	56.4	20.1	0.557	10.38	Control
" 30–31	11.25	305	32.5	33.1	23.6	0.525	9.88	α-Estradiol†
" 31–Apr. 1	11.35	310	39.5	41.7	15.6	0.428	10.13	Control
Apr. 1–2	11.50	305	37.1	41.4	22.0	0.394	8.22	"
" 2–3	11.65	440	60.6	57.7	19.4	0.578	10.93	"
" 3–4	11.65	425	57.6	58.6	17.5	0.527	12.06	"
" 4–5	11.65	515	73.0	67.8	17.0	0.515	11.42	"
" 5–6	11.45	510	73.2	64.4	15.4	0.540	11.45	"
" 6–7	11.35	410	50.6	49.0	16.3	0.478	9.69	"
" 7–8	11.45	410	56.1	56.2	20.5	0.566	11.32	"

* Throughout this study the animal was fed a constant diet which contained 62 m.eq. of sodium, 63 m.eq. of chloride and 30 m.eq. of potassium per day.
† A single injection of 5 mg. of α-estradiol in 3 cc. of mazola oil.

Following the period of decreased renal excretion there usually was observed a corresponding period in which an increased excretion of all the substances retained was noted. The action of estrone and α-estradiol differed from that of the cortin-like substances and progesterone in the more prolonged period of sodium and chloride retention and in the marked retention of total nitrogen and inorganic phosphorus.

In male suprarenalectomized dogs injections of amniotin (20,000 to 100,000 I.u.) partially prevented the increased renal excretion of sodium and chloride which follows withdrawal treatment by supra-

TABLE IV

A Comparison between the Effectiveness of Amniotin and Suprarenal Cortical Extract in Preventing Sodium and Chloride Diuresis in Suprarenalectomized Dogs (2, 4)*

Period	Urine volume cc.†	Sodium m.eq.†	Chloride m.eq.†	Treatment
				Dog 2
4 days	630	94.7	91.6	No treatment
4 days	540	73.9	63.6	Amniotin‡ only
4 days	490	57.0	62.6	Suprarenal cortical extract§ only
				Dog 4
4 days	630	80.1	69.1	No treatment
6 days	630	68.0	60.9	Amniotin only‖
6 days	560	49.3	55.8	Suprarenal cortical extract§ only

* Throughout this study the animals were fed a constant diet which contained 62 m.eq. of sodium, 63 m.eq. of chloride and 30 m.eq. of potassium per day.

† These values represent average daily excretions.

‡ 20,000 International units of amniotin were injected subcutaneously daily.

§ Suprarenal cortical extract, 4 to 5 cc. (160 to 200 gm. of cortex) injected subcutaneously twice daily.

‖ 240,000 International units of amniotin were used; 100,000 units on the 1st day, 60,000 on the 3rd day, 40,000 on the 4th day and 40,000 on the 6th day.

TABLE V

The Effect of α-Estradiol on the Renal Excretion of Electrolytes in Patient C. N.*
(Addison's Disease)

24 hour period	Body weight kg.	Urine volume cc.	Sodium m.eq.	Chloride m.eq.	Potassium m.eq.	Inorganic phosphorus gm.	Total nitrogen gm.	Treatment
May 6–7	53.4	880	207.0	212.8	29.5	0.233	6.18	Suprarenal cortical extract†
" 7–8	53.4	980	230.2	247.2	48.5	0.273	6.37	Estradiol‡
" 8–9	54.0	890	184.2	198.9	33.3	0.198	5.49	Control
" 9–10	54.3	880	167.5	173.9	32.5	0.257	5.10	"
" 10–11	54.5	940	156.4	156.5	28.4	0.167	3.85	"
" 11–12	54.1	920	205.8	222.2	37.5	0.271	4.20	"
" 12–13	53.8	1500	327.0	323.3	59.4	0.632	7.40	"

* Throughout this study the patient was maintained on a constant diet which contained 305.4 m.eq. of sodium, 307.0 m.eq. of chloride, 80.2 m.eq. of potassium, 1.23 gm. of phosphorus and 10.95 gm. of nitrogen per day.

† Suprarenal cortical extract, 2 cc. (80 gm. of cortex) injected subcutaneously twice daily.

‡ A single subcutaneous injection of 17 mg. of α-estradiol in 5 cc. of mazola oil.

renal cortical extract (Table IV). However, the quantities of amniotin injected were not effective in preventing the gradual onset of symptoms of suprarenal insufficiency. In a female patient with Addison's disease (C.N., Table V), the subcutaneous injection of 17 mg. of α-estradiol resulted in a marked and prolonged retention of sodium, chloride, inorganic phosphorus and total nitrogen. This effect was similar to that observed in normal male dogs.

Testosterone Propionate

The effect of testosterone propionate was observed in three normal dogs, one suprarenalectomized dog and one male human subject.

TABLE VI

The Effect of Testosterone Propionate on the Renal Excretion of Electrolytes in a Normal Dog (5)*

24 hour period	Body weight	Urine volume	Sodium	Chloride	Potassium	Inorganic phosphorus	Total nitrogen	Treatment
1938	*kg.*	*cc.*	*m.eq.*	*m.eq.*	*m.eq.*	*gm.*	*gm.*	
May 16–17	15.70	550	51.9	57.1	22.1	0.500	9.62	Control
" 17–18	15.75	420	48.2	39.2	23.6	0.396	9.53	Testosterone propionate†
" 18–19	15.75	650	56.4	64.7	14.3	0.460	9.34	Control
" 19–20	15.85	580	44.5	57.9	21.7	0.449	9.28	"
" 20–21	15.90	705	67.1	72.0	23.3	0.482	9.47	"

* Throughout this experiment the animal was maintained on a constant diet which contained 62 m.eq. of sodium, 63 m.eq. of chloride and 30 m.eq. of potassium per day.

† A single subcutaneous injection of 125 mg. of testosterone propionate in 5 cc. of sesame oil.

In the normal dogs a single subcutaneous injection of 25 mg. of testosterone propionate in 1 cc. of sesame oil had no appreciable effect on the 24 hour renal excretion of electrolytes. A single subcutaneous injection of 125 mg. of testosterone propionate in 5 cc. of sesame oil resulted in a very slight decrease in the renal excretion of sodium and total nitrogen (Table VI). However, the urine volume and the renal excretion of chloride and inorganic phosphorus were markedly reduced. The maximum effect of testosterone propionate

appeared to occur during the 24 hour period immediately following injection. A second decrease in the renal excretion of sodium was observed during the 48 to 72 hour period after injection. The large quantity (125 mg.) of testosterone propionate required to produce this change would indicate that testosterone had little specific effect on the renal excretion of electrolytes.

The effect of repeated daily injections of smaller quantities (25 mg.) of testosterone propionate was observed in one normal male dog and in one intact human male subject. In the dog 25 mg. of testosterone

TABLE VII

The Effect of Repeated Injections of Testosterone Propionate on the Renal Excretion of Electrolytes in a Normal Dog (6)*

Period	Body weight	Urine volume	Sodium	Chloride	Potassium	Inorganic phosphorus	Total nitrogen	Treatment
	kg.	cc.†	m.eq.†	m.eq.†	m.eq.†	gm.†	gm.†	
1938						0.590	11.35	Control‡
Jan. 11–15	9.10	635	55.5	61.8	18.7	0.556	10.25	Testosterone§
" 15–22	9.70	655	56.5	63.6	11.6	0.410	7.23	Control
" 22–25	9.75	660	54.9	56.8	12.9	0.813	12.05	"
" 25–28	9.75	700	61.6	62.5	17.7	0.546	10.58	"
" 28–Feb. 1	9.90	580	55.3	54.3	13.4			

* Throughout this study the animal was fed a constant diet which contained 62 m.eq. of sodium, 63 m.eq. of chloride and 30 m.eq. of potassium per day.
† The values in the table represent the average daily excretion.
‡ An equivalent quantity of a sterile solution of oil was injected subcutaneously.
§ 25 mg. of testosterone propionate in 1 cc. of sesame oil was injected daily.

propionate in 1 cc. of sesame oil injected daily for 7 days resulted in an increase in body weight and a marked decrease in the renal excretion of inorganic phosphorus, total nitrogen and potassium (Table VII). This effect reached its maximum during the 3 day period following the last injection of testosterone propionate. An increased excretion of potassium, total nitrogen and inorganic phosphorus occurred during the subsequent 3 day period. Throughout this experiment little effect was observed on the renal excretion of sodium and chloride.

In the human subject daily intramuscular injections of 25 mg. of

testosterone propionate were made for 7 days. During the period of treatment and for 3 days following, a decreased renal excretion of potassium, inorganic phosphorus and total nitrogen was observed
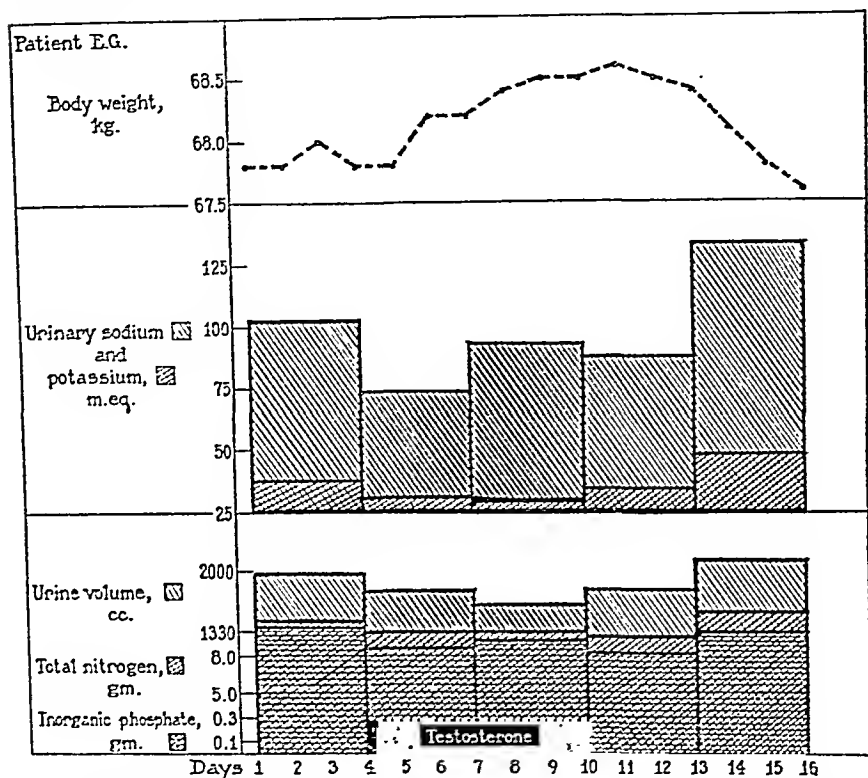
CHART 1. The effect of repeated daily intramuscular injections of testosterone propionate on the renal excretion and body weight of a male subject (E. G.).

An intramuscular injection of 25 mg. of testosterone propionate in 1 cc. of sesame oil was given daily. During the control periods an intramuscular injection of 1 cc. of sesame oil was given daily.

Throughout this study the patient was maintained on a constant diet which contained 89.2 m.eq. of sodium, 84.0 m.eq. of potassium, 1.75 gm. of phosphorus and 15.6 gm. of nitrogen per day.

(Chart 1). This effect was similar to that noted in the normal dog. In subject E.G. treatment with testosterone propionate also resulted in a marked decrease in the renal excretion of sodium and chloride and an increase in body weight. 72 hours after injections of testo-
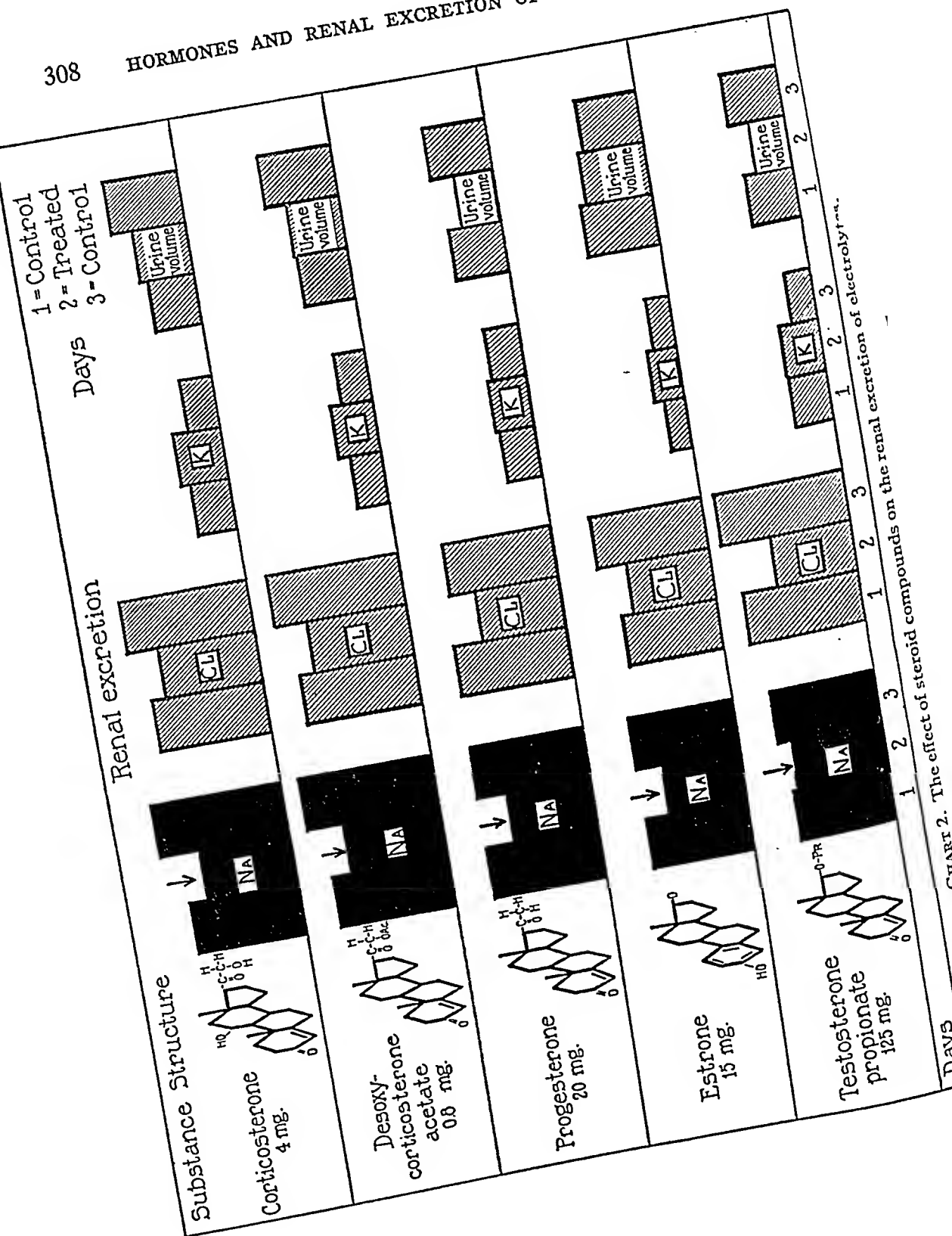
CHART 2. The effect of steroid compounds on the renal excretion of electrolytes.

sterone propionate were discontinued a marked diuresis occurred. At this time marked weight loss and an increased renal excretion of sodium, chloride, potassium, inorganic phosphorus and total nitrogen were noted.

For a period of 6 days a single daily subcutaneous injection of 25 mg. of testosterone propionate in 1 cc. of sesame oil was substituted for suprarenal cortical extract in a suprarenalectomized male dog. The typical sodium and chloride diuresis which occurs immediately after the withdrawal of suprarenal cortical extract was not influenced by the treatment with testosterone propionate. During this period the animal lost weight and strength; there was an elevation of the blood non-protein nitrogen and the animal appeared to be developing the symptoms of suprarenal insufficiency.

A comparison of the action of corticosterone, desoxy-corticosterone acetate, progesterone, estrone and testosterone propionate on the renal excretion of sodium, chloride and potassium has been made in Chart 2.

A number of other compounds have been injected into normal dogs without producing any appreciable effect upon the renal excretion of electrolytes: androstenediol (40 mg.), androstenedione (40 mg.), 1:2-cyclopentenophenanthrene (50 mg.), cholesterol (200 mg.) purified through dibromide, p-hydroxy-propenyl benzene (200 mg.) and 7-keto-cholesteryl acetate (500 mg.).

DISCUSSION

The sex hormones thus far tested, have shown a marked variation in their effect on the renal excretion of electrolytes. A single subcutaneous injection of 20 mg. of progesterone resulted in a decreased renal excretion of sodium and chloride approximately equivalent to that which followed the injection of 0.8 mg. of desoxy-corticosterone acetate or 4 mg. of either corticosterone or 11-dehydro-corticosterone. In similarity with the action of the cortin-like compounds but in contrast to the action of estrone, α-estradiol and testosterone propionate, progesterone (20 mg.) had no appreciable effect on the renal excretion of inorganic phosphorus or total nitrogen. The duration (24 hours) of the decreased renal excretion of sodium and chloride following the injection of progesterone was also similar to that observed after the injection of the cortin-like compounds. The effect

of progesterone differed from corticosterone, 11-dehydro-corticosterone and desoxy-corticosterone acetate in the absence of an increased excretion of sodium and chloride (rebound) during the second 24 hour period.   The injection of progesterone was followed by a slight rise in potassium excretion which in no instance was as striking as that following the injection of desoxy-corticosterone acetate.

A study of the effect of progesterone on the renal excretion and survival of the suprarenalectomized dog has been limited by the supply of progesterone.   A single subcutaneous injection of progesterone (20 mg.) prevented the characteristic sodium and chloride diuresis which occurs during the first 24 hours after treatment with suprarenal cortical extract is discontinued.   This effect, like that of the cortin-like substances, lasted only 24 hours.   In the dosage used, progesterone did not appear to be toxic to the suprarenalectomized dog.   The question of whether progesterone in adequate doses (20 mg. or more per day) can be substituted for the cortin-like compounds in maintaining suprarenalectomized animals must be left unanswered until larger quantities of progesterone are available.   The injection of estrone (15 mg.) α-estradiol (5 mg.) was followed by a marked decrease in the renal excretion of sodium, chloride, inorganic phosphorus and total nitrogen.   This action differed in two respects from the action of the cortin-like substances and progesterone: (a) The period of decreased renal excretion of sodium and chloride was more prolonged, and (b) a quantity of the compound sufficient to affect the renal excretion of sodium and chloride usually resulted in an appreciable retention of inorganic phosphorus and total nitrogen. A slight rise in the renal excretion of potassium was noted on the day of injection.

Although the sodium and chloride diuresis following the withdrawal of treatment by suprarenal cortical extract in a suprarenalectomized male dog was reduced by injections of amniotin, the onset of symptoms of suprarenal insufficiency was not prevented.   Such an experiment does indicate, however, that the mechanism of sodium and chloride retention induced by the female sex hormones does not necessarily have to be mediated through the suprarenal cortex.   The sodium and chloride retention which occurred in a female patient with Addison's disease following the injection of 17 mg. of α-estradiol would appear

to confirm this.   There is no evidence to date that repeated injections of estrogenic substances are either beneficial or desirable in the treatment of suprarenal insufficiency.

The sodium and chloride retaining effect of testosterone propionate was quite feeble in comparison to the cortin-like substances, progesterone, estrone and α-estradiol.   Large quantities of testosterone propionate (125 mg.) were necessary to produce an appreciable change in the renal excretion of sodium and chloride in normal dogs.   Repeated daily injections of 25 mg. of testosterone propionate in a normal dog had slight effect on the renal excretion of sodium and chloride but did produce an appreciable retention of potassium, inorganic phosphorus and total nitrogen.

Repeated daily injections of testosterone propionate (25 mg.) in a human male subject resulted in a decreased renal excretion of sodium and chloride as well as potassium, inorganic phosphorus and total nitrogen.   This retention persisted for a period of 72 hours after treatment was discontinued.

Daily injections of testosterone propionate (25 mg.) were not effective in preventing the sodium and chloride diuresis which follows withdrawal of treatment by suprarenal cortical extract in suprarenalectomized dogs.   No beneficial effect was noted on the survival period of such animals.

SUMMARY

In normal male dogs subcutaneous injections of progesterone, estrone, α-estradiol or testosterone propionate were followed by a decreased renal excretion of sodium and chloride.   The compounds differed markedly in their potency and in the duration of the effect following a single subcutaneous injection.   The injection of estrone, α-estradiol or testosterone propionate was followed by a decreased renal excretion of inorganic phosphorus and total nitrogen.   On the day of injection a slight increase in the renal excretion of potassium frequently followed administration of progesterone, estrone, α-estradiol or testosterone propionate.   Experiments on suprarenalectomized dogs indicated that the effect of the sex hormones on the renal excretion of electrolytes was not necessarily mediated through the suprarenal gland.   With the possible exception of progesterone

none of the compounds studied was effective in prolonging the life of suprarenalectomized male dogs.

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PROPAGATION OF THE VIRUS OF HUMAN INFLUENZA IN THE GUINEA PIG FETUS*

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The reports of Woolpert and associates (1–4) have called attention to the use of the mammalian fetus as an experimental animal for the study of various infectious agents.   It has been pointed out that the fetus is frequently more susceptible to a particular agent than the postnatal animal of the same species, and furthermore is originally free of bacteria.   Since viruses especially are known to flourish in embryonic cells, it would be expected that fetal animals would constitute a good medium for the propagation of viruses.   This has been found true for the vaccinia virus (3), the guinea pig submaxillary virus (4), and the herpes virus (5) in the fetal guinea pig, and the vaccinia virus (6) in the fetal rabbit.

For several reasons it seemed of interest to investigate the possibility of propagating the influenza virus in the fetal guinea pig. In the first place, guinea pigs as a species appear to be fairly resistant to this virus.   Although McIntosh and Selbie (7) isolated a virus from the lung tissue of an influenza patient which could be transmitted through guinea pigs in series, causing fever and sometimes death, it is not clear that they were dealing with what we now recognize as the virus of human influenza.   Stuart-Harris (8) was able to infect young guinea pigs in series by intranasal inoculation of a virulent ferret strain of the human influenza virus, but only subclinical infections were induced.   The virus apparently proliferated and persisted in turbinate tissue only, since the lungs were shown to be free of virus. Our own experience and that of others indicate that the guinea pig is relatively insusceptible to this virus.   The natural question arises: Does the fetus share this species resistance?

Furthermore, assuming that the fetal guinea pig should prove susceptible to the influenza virus, it is of interest to inquire what the distribution of the virus would be in fetal tissues. In postnatal animals and man the virus is infectious principally, if not only, by way of the respiratory tract and is recoverable chiefly from tissues or secretions of the respiratory system. This holds true despite the findings of Smorodintseff and Ostrovskaya (9) that small amounts of virus can be detected by special means in other organs and fluids of inoculated mice. Will this evident pneumotropism be manifested also in the fetal animal, an animal in which the respiratory system is non-functioning and which, for practical reasons, must be inoculated by routes other than the intranasal?

Other considerations of interest in such work concern possible modification of the virus by passage through fetuses, and evaluation of the fetus as a potential experimental animal for the detection of small amounts of virus, or for the production of large quantities of virus free of bacteria.

Materials and Methods

Strain of Virus.—We employed the PR8 strain of human influenza virus isolated by Francis in 1934 (10) and kindly supplied us by him. It was in its 158th mouse passage when received.

Maintenance of Virus.—The stock virus has been maintained in our laboratory by passage through mice in the usual manner. For this purpose young Swiss mice are inoculated intranasally under light ether anesthesia and their lungs are harvested for virus 48 hours later. Formerly the whole lungs were stored in 50 per cent glycerine at ordinary refrigeration temperature and 10 per cent suspensions were made in physiological saline as needed for subinoculation. More recently we have made suspensions directly in saline and stored the suspensions at $-78°C$. in a thermos jug containing alcohol and solid CO_2 as suggested by Turner (11).

Preparation of Virus for Inoculation into Fetuses.—Since a bacteriologically sterile virus was required for fetal inoculation, the mouse lung tissue could not be used directly. We employed, therefore, either Berkefeld V filtrates of mouse lung virus, tissue culture virus derived from the stock strain, or mouse fetus virus (12) derived from the same source. There appeared to be no difference in the results obtained with virus from the various sources.

Guinea Pigs.—Pregnant guinea pigs of approximately 35 days gestation age were selected as needed from the breeding stock of the University Farms. Through

experience the fetal age was judged with a fair degree of accuracy by palpation of the mother, and was determined with precision by weighing the fetuses at the termination of the experiment. The guinea pigs were a hybrid assortment purchased by the Farms in small lots from various producers.

Technique of Inoculating the Fetuses and Recovering the Virus.—This has been previously described (1). It consists essentially in surgical exposure of the gravid uterus and inoculation of the fetus by needle puncture through the uterine wall and fetal membranes. In these experiments the inoculations were made intracerebrally in 0.05 to ·0.10 cc. amounts. The fetuses were removed by cesarean section 2 to 7 days later, at which time gross examination was made and tissues were taken for virus determination and histologic preparation. Ether vapor anesthesia (13) was used for all operative work.

Tests for Virus.—Qualitative and quantitative tests for virus were made in young white Swiss mice, inoculated in the usual manner. Surviving test mice were sacrificed at the 10th day, or in some instances were used in cross-immunity studies. The sera for cross-neutralization tests were prepared by subcutaneous and intravenous immunization of rabbits and the tests were made in mice by intranasal inoculation of the serum-virus mixtures which had been allowed to stand for 30 to 60 minutes at 37°C., sometimes with an additional incubation for 12 hours at 4°C.

Transmission of the Influenza Virus to Fetal Guinea Pigs and Distribution of Virus in Fetal Tissues

In numerous instances an influenza virus infection was established in the guinea pig fetus by intracerebral administration of the virus. In the absence of occasional technical difficulties, transmission to the fetus was accomplished whenever a bacteriologically sterile virus of proven potency for mice was employed, regardless of the immediate source of the virus, whether mouse lung filtrate, tissue culture, mouse fetus, or guinea pig fetus. Table I shows the distribution and titer of virus in several tissues of a fetus 48 hours after the inoculum had been introduced intracranially. It is seen that the highest yield of virus appeared in lung tissue and that placenta and liver were also good sources of virus, whereas blood was of low titer, and no virus was demonstrable in the brain at the dilution (1–10) tested, even though it was the organ receiving the original inoculum. In general these results are typical of numerous similar determinations, except that the brain occasionally contained small amounts of virus.

Serial Passage of the Influenza Virus through Fetal Guinea Pigs

Several series of passages were accomplished by inoculating the fetuses intracerebrally and harvesting the fetal lungs for passage virus 48 hours later. The lung tissue was usually prepared for sub-inoculation as a 10 per cent suspension in saline. In one series the virus was carried through 10 transfers, in another through 16. Several shorter series were terminated because of the lack of suitable animals. The A series of 10 transfers, the first attempted, is shown in Table II. It was initiated with virus from the 2nd transfer in a mouse fetus series. The table indicates the distribution of virus in certain fetal tissues at progressive stages of serial passage and following different

TABLE I

Distribution and Titer of Influenza Virus (Human) in Fetal Guinea Pigs as Determined by Intranasal Inoculation of Adult Mice*

Fetal tissue†	Dilution of tissue				
	Undiluted	1–10	1–100	1–1000	1–10,000
Lung	—	3/3	3/3	2/3	2/3
Placenta	—	3/3	2/3	1/3	0/3
Liver	—	2/3	0/3	0/2	0/3
Blood	2/3	0/3	0/3	0/3	0/3
Brain	—	0/3	0/3	0/3	0/3

* The figures show the number of mice dying of those inoculated.
† Tissues of a fetus of the 13th serial passage (K XIII).

incubation periods. It may be seen that, with rare exceptions, the lung tissue yielded virus of good titer throughout the series and after the several incubation periods. It is perhaps significant that virus was recovered from the brain at the first passage but could not usually be demonstrated in that organ in later transfers. Judging the potency of the virus from the survival time of test mice, it may be inferred that the conditions chosen for propagation of the virus through fetuses were favorable, and that the potency of the virus was maintained without significant alteration.

Table III depicts the K series of passages in which the original inoculum was a Berkefeld V filtrate of a suspension of infected lungs

of adult mice. This table records tests for virus in the fetal lungs throughout the series of 16 transfers, indicating the day of death for the test mice and the approximate percentage involvement of the

TABLE II

Distribution of Influenza Virus (Human) in Fetal Guinea Pigs at Progressive Stages of Serial Passage

Passage No.	Length of incubation in fetus	Tests in adult mice Tissue tested* and day of death of test mice			
		Lung	Brain	Liver	Placenta
	days				
A I	2	3, 3, 3	5, 6, 10		
	4	3, 3, 3	5, 6, 7		
	6	4, 5, 6			
A II	2	4, 4, 4		4, 5, 6	
	3	3, 6			
A III	2	4, 4, 5			
	4	S, S			
A IV	2	S, S, S, S	S, S, S	S, S	
A V	2	4, 5, 6	S, S, S		
	4	S, S	S, S		
A VI	2	3, 5, S	S, S	S, S	
A VII	2	4, 4, 6		7	
A VIII	2	4, 4			
A IX	2	4, 4, 5	7, S, S	4, 6, 6	4, 4, 4, 5
	4	4, 4, 5, 6		5	
A X	2	3, 4, 5, 5			

S = survived.
* In 10 per cent suspension.

mouse lungs. The table brings out a point not previously emphasized, namely, that the fetal guinea pig can be infected with amounts of virus which produce little if any

starting with such material one may obtain virus of good titer after passing it through one or two fetuses. This is illustrated by the falling off in titer, for one reason or another, at the 10th, 12th, and 15th passages, with a restoration to full potency in each case on further

TABLE III

Tests for the Influenza Virus (Human) in Lungs of Fetal Guinea Pigs Inoculated in Serial Passage

Date	Passage No.	Effects in test mice	
		Day of death	Lung consolidation Per cent involved in the gross
1937–38			
Oct. 20	K I	2, 3, 3, 3, 3, 3	60, 100, 80, 90, 90, 100
" 29	K II	3, 4, 4, 4, 4, 6	80, 90, 100, 100, 80, 80
Nov. 5	K III	3, 4, 4	90, 100, 100
" 11	K IV	3, 3, 4	90, 50, 100
" 17	K V	4, 4, 4	90, 100, 100
" 19	K VI	2, 3, 3	60, 90, 100
" 24	K VII	5, 5	80, 100
Dec. 1	K VIII	3, 3	80, 100
" 6	K IX	5, 6	100, 100
" 16	K X	Im,* Im, Im	—
" 22	K XI	7, 8, Im	80, 100, —
Jan. 9	K XII	3, 4, 4	95, 100, 100
Mar. 10	K XII (retested)	Im, Im, Im	—
" 11	K XIII	9, Im	100, —
" 13	K XIV	7, 7, 7, 8, 8	100, 90, 100, 100, 100
" 23	K XV	3, 4, 4	100, 95, 100
Apr. 15	K XV (retested)	Im, Im, Im	—
" 15	K XVI	3, 4, 5	100, 100, 80

* Im = not killed, but immunized to the virus, as shown by later tests with potent material.

passage of the virus through fetuses. For example, K XII virus was lethal for mice when it was first tested (January 9), but was capable only of immunizing mice when retested after 2 months storage (March 10). Each of the next 3 transfers enhanced its titer until it was again killing mice in 3 to 4 days.

Properties of the Passage Virus

It was of course pertinent to inquire whether the virus which had been carried through several transfers in fetal guinea pigs actually represented the influenza virus and whether its properties had undergone significant modification.

To determine the identity of the virus, cross-immunity (Table IV) and cross-neutralization tests (Tables V and VI) were carried out, using the mouse passage strain of virus as a basis of reference. For the cross-immunity tests, we used mice which had survived previous exposure to virus in other experiments. In some instances their resistance to the homologous virus had been confirmed and enhanced by reinoculation. Those mice which had survived inoculation with mouse passage virus were immune to the fetal guinea pig strain, and the converse was also true (Table IV).

In the cross-neutralization experiments, each of the two strains of virus was tested after mixture with saline, with normal rabbit serum, with the homologous rabbit antiserum, and with the heterologous antiserum, *i.e.*, serum of a rabbit immunized to the other strain. Since all tests were not carried out at one time, a duplication of the virus-saline control tests appears in both tables. Table V shows the results of testing the mouse passage strain against homologous and heterologous sera; a complete cross-neutralization was demonstrated. In Table VI are recorded the findings obtained in a comparable manner for the fetal strain. At first glance there appears to be an anomaly here, in that the fetal virus has been completely neutralized by heterologous serum but not by homologous serum. This was probably due to the fact that in the tests with homologous serum a stronger virus was employed, and accordingly neutralization by serum was somewhat masked. It may be seen that this virus when inoculated with saline or with normal serum killed within 3 days all of the mice tested, and that when it was given with homologous serum, death was deferred until the 6th, 7th, or 8th day. On the other hand, the lot of fetal virus used in tests against heterologous serum did not kill under 7 days and was evidently readily neutralized by the serum used at that time.

All of the fetal virus used in these cross-immunity and cross-neutralization experiments was from the K series of transfers (Table III)

TABLE IV

Cross-Immunity Tests in Mice: Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human)

History of mice	Tested with	Mice dying of those inoculated
Survived inoculation with mouse virus	Fetal guinea pig virus	0/8
Normal controls	Fetal guinea pig virus	4/4
Survived inoculation with fetal guinea pig virus	Mouse virus	0/13
Normal controls	Mouse virus	5/5

TABLE V

Cross-Neutralization Tests of Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human) in Adult Mice

I. Mouse Strain against Homologous and Heterologous Sera

Inoculum	Mice dying of those inoculated	Day of death
Mouse virus + saline	5/6	3, 4, 5, 7, 9
Mouse virus + normal rabbit serum	6/6	3, 5, 5, 6, 6, 6
Mouse virus + heterologous antiserum	0/6	—
Mouse virus + saline	3/3	4, 4, 4
Mouse virus + homologous antiserum	0/3	—

TABLE VI

Cross-Neutralization Tests of Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human) in Adult Mice

II. Fetal Guinea Pig Strain against Homologous and Heterologous Sera

Inoculum	Mice dying of those inoculated	Day of death
Fetal virus + saline	3/3	3, 3, 3
Fetal virus + normal rabbit serum	6/6	3, 3, 3, 3, 3, 3
Fetal virus + homologous antiserum	6/6	6, 7, 7, 7, 8, 8
Fetal virus + saline	4/4	7, 7, 7, 8
Fetal virus + heterologous antiserum	0/5	—

and principally from the latter transfers in that series, that is, after a certain stabilization of the passage strain was assured. The results of these tests indicate therefore that the fetal passage strain was immunologically similar to or identical with the mouse strain from which it was derived.

As to other properties of the passage strain, we have studied only its behavior in routine manipulation in the laboratory. On this basis there was no evidence that changes in virulence or specificity of the virus had been induced. This was inferred from the observation of its pathogenicity for mice, the titers obtained, the incubation periods, the behavior in storage, etc.

Throughout serial passage the guinea pig fetal strain has remained bacteriologically sterile.

Effects of the Influenza Virus on the Guinea Pig Fetus

Although the influenza virus disseminated widely in the fetuses, there was little gross evidence of damage to fetal tissues. The fetuses, with rare exceptions, were living when examined 48 hours after inoculation. Death was occasionally observed after the longer incubation periods (4 to 6 days), and its occurrence was irregular. Details of the pathological changes encountered in these animals will be reported in a separate communication (14).

A particular study of the mother guinea pigs which carried the fetuses throughout the experimental period was not made, but no symptoms or lesions attributable to the virus infection of the fetuses were observed in the mothers.

DISCUSSION

It seems apparent, on the basis of the data recorded, that the influenza virus actually multiplied in the tissues of the fetal guinea pig. This may be inferred from a consideration of the quantitative relationship of virus inoculum to virus yield, especially in the serial passage experiments wherein there was no loss of titer throughout a number of transfers, although an enormous dilution of the original inoculum was effected. Multiplication of the virus is implied also by the distribution of virus in particular tissues following intracerebral inoculation. It seems probable that actual multiplication occurred

in those organs which were found to contain large quantities of virus, specifically, lung, liver, and placenta, and that these parts were seeded from the brain through the blood stream. However, it is evident that the virus did not circulate freely in blood channels, because it was actually detectable there only in small amount, and because its titer in the various organs differed widely and generally exceeded that in the blood. It is of interest that the brain which received the original inoculum appeared incapable of supporting growth of the virus, whereas the virus apparently found in the lung an environment favorable for proliferation. This is the more striking in that the lung is non-functioning in the fetal animal, and the invasion of this tissue reemphasizes the specific association of the influenza virus and the pulmonary tract. The high virus content here is coincident with the greatest histologic change in any fetal part (14). Evidently, the fetal lung of this relatively insusceptible species shows the same specific receptivity as the lung of susceptible animals.

It should be pointed out, however, that we may have encouraged the pneumotropic[1] proclivities of the virus by employing lung tissue for subinoculation in serial passage. It is consistent with this suggestion that a fair quantity of virus was found in brain tissue in the first passage of series A (Table II), and little or no virus in the same tissue in subsequent stages of the series. Detailed confirmation of this finding would be necessary before conclusions would be warranted. It would be of interest to determine whether the virus could be passed in series by using brain tissue for subinoculation, and if so, whether modification of the virus would be induced thereby.

Although no route of inoculation other than the intracerebral was used in these experiments, there is reason to suppose that infection through other channels could be accomplished. The conditions for serial passage of the virus, viz., an incubation period of 48 hours and the use of lung tissue for subinoculation, were dictated by reasoning from analogy on the basis of the reactions of adult mice to the in-

[1] We have retained the term pneumotropic because of previous usage. We do not imply or believe that the virus is attracted to the lungs selectively; we wish to emphasize only that lung tissue constitutes a relatively favorable medium for growth of the virus.

fluenza virus. These conditions were found satisfactory at the outset and were consistently employed throughout the work. Presumably the series could have been continued indefinitely in this way, although it may be that modifications of this technique might have proved equally or more favorable.

It would be of interest to know whether passage of the influenza virus through the fetal guinea pig might adapt it to that species to the degree that it would produce in the postnatal animal a clinical infection. We have carried out a few experiments with this hypothesis in mind but the results have been inconclusive thus far.

SUMMARY

The PR8 strain of human influenza virus was found to proliferate and disseminate widely in the tissues of fetal guinea pigs inoculated *in utero*. Large quantities of virus free of bacteria were recovered from lung, liver, and placenta, and smaller quantities from blood and brain, after incubation periods ranging from 2 to 6 days. Although the fetuses proved to constitute an excellent medium for the propagation of influenza virus, they evinced little gross reaction to the infection.

Several series of passages from fetus to fetus were accomplished; one consisted of 10 transfers, another of 16. For serial passage the virus was inoculated intracerebrally into half-grown fetuses and the fetal lungs were harvested 48 hours later as a source of virus for subinoculation. It is concluded that multiplication of the virus occurred particularly in the lungs, which may be considered a significant reaffirmation of the pneumotropic tendencies of this virus.

Following passage in series the virus was found, on the basis of cross-immunity and cross-neutralization tests, to be immunologically identical with the mouse passage virus from which it was derived. Other properties also appeared to be unaltered by passage of the virus under these conditions.

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ALTERED CUTANEOUS CONDITIONS IN THE SKIN OF TUBERCULOUS GUINEA PIGS AS DEMONSTRATED WITH A VITAL DYE

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PLATE 10

The questions that arise concerning allergic reactions in the skin consequent on infection illustrate the inadequacy of our knowledge of the mechanisms involved. A recent study of reactions in guinea pigs after dermal inoculations with tubercle bacilli, carried out in connection with the preparation for publication of some experiments of the late Henry Sewall (1), prompted us to a further study of the skin. By the use of a dye relatively inert in the normal animal, we have found that in the tuberculous guinea pig there is a restriction to the spread of this material after intradermal injection. There is reason to suppose that this property of restriction of spread in a state known to be allergic may be a factor in the reaction of the skin to tuberculin.

Tuberculosis is the disease in which the study of dermal reactions has been most comprehensive and a proper understanding of the nature of such reactions is of increasing significance in connection with recent clinical surveys. The guinea pig has been the animal most used in the experimental work, since it reacts readily to small amounts of tuberculin after infection. This reaction to tuberculin is generally conceded to be specific. The type of the reaction is characterized by a delayed erythema and edema and, in more highly reactive states, by induration and necrosis. It differs from the Arthus reaction induced experimentally, in being more delayed in appearance and less transient, in that hemorrhage is not prominent, in the lack of correlation between the intensity of the dermal reaction and the antibody titer of the blood, and in that passive transfer has not been accomplished.

Dienes (2) has shown that the tuberculous guinea pig, when sensitized to a non-bacterial protein, such as egg white, reacts differently from a normal animal so sensitized. The skin reaction in the tuberculous-sensitized animal becomes more like a tuberculin reaction and after an intravenous injection of egg white the animal passes into a condition like tuberculin shock. In tuberculin shock an animal shows a gradual and profound fall in temperature and, as Dienes has found, a hypoglycemia leading in severe sensitization to death after several hours. Lurie (3) has shown that tuberculous animals, as illustrative of a phase of immune reactions, vary from the normal in their power to restrict the spread of foreign materials, such as agar and India ink. A study of the Arthus phenomenon by Opie (4) has demonstrated that when horse serum is injected intradermally into rabbits sensitized to it the protein remains localized at the point of injection, and the degree of the dermal reaction to the protein is correlated with the antibody titer in the serum. Freund, Laidlaw, and Mansfield (5) have shown that the sensitivity in the tuberculous animal differs from the Arthus reaction studied by Opie in that when the reaction of the skin to tuberculin disappears, there is no loss of the complement-fixing antibody titer. Bordet (6) and Freund (7) have shown that tuberculous animals are more susceptible to heterologous bacterial filtrates than normal animals. These observations justify the supposition that tuberculous animals have an altered condition of the skin with respect to specific and non-specific foreign material.

Hudack and McMaster (8) have made an extensive study of diffusion in the skin by means of the use of non-toxic "vital" dyes. They showed that practically every true dermal injection is both interstitial and intralymphatic. The mesh of the dermal lymphatic plexus is so close that the needle cuts through some of the vessels and the dye enters the open ends under pressure. By making a minute hole in the skin and then introducing extremely small amounts of dye into this space, Parsons and McMaster (9) have been able to make pure interstitial injections and thus have obtained material in which to study both the physiologic factors of diffusion and of normal lymphatic drainage.

In the present study, we have found that there is a restriction both to the diffusion of dye in the connective tissue spaces and to the drainage of the same dye into the lymphatics in guinea pigs in two different infections, namely, tuberculosis and epizootic lymphadenitis.

Materials and Methods

Guinea pigs inoculated with stock cultures of tubercle bacilli, strains H-37 and A-14, by Dr. K. C. Smithburn were employed as the tuberculous animals. The strain A-14 was received from Dr. E. R. Long of The Henry Phipps Institute, Philadelphia. Normal controls were stock guinea pigs apparently free from disease. For comparison with the reactions in tuberculosis, guinea pigs with epizootic lymphadenitis were used, the disease having been diagnosed by palpa-

tion of the enlarged lymph nodes and by a skin test with a bacterial extract furnished us by Dr. J. K. Moen (10). He has shown that animals reacting to this bacterial extract either have at the time, or have had recently, an infection with a hemolytic Streptococcus, Lancefield type C.

Areas of skin to be studied were prepared by several different methods. The hair was clipped and then shaved with a razor, or the hair was removed with an epilator or by close clipping with an electric animal clipper. The majority of the animals were prepared by the latter method 24 hours before the injections. Studies were made of the spread of dye after these different methods of preparation of the skin and no differences were observed. The material used for injection was a diazo dye, pontamine sky blue, which we received from Dr. McMaster, to whom we are indebted for assistance in our methods as well. The dye had been dialyzed in running water until free from salts and then made into a 2.5 per cent, approximately isotonic solution. A Dewitt and Herz syringe was used, equipped with a threaded plunger with an adjustable nut which can be set so as to limit the amount of fluid injected to amounts as small as 0.001 cc. with reasonable accuracy. It had a Luer tip for the needles which were No. 29 and were ½ inch in length. A new syringe was used which had never been filled with any products from tubercle bacilli. The amount of dye injected was as close to 0.025 cc. as possible. Injections were performed under a dissecting microscope under a bright light. The needle was inserted just beneath the epidermis and the syringe was held parallel to the body of the animal. By exerting gentle pressure the point of the needle was introduced about a distance of 2 mm. The skin over the point of the needle was then covered with mineral oil to facilitate observation of the dye. The dye was then injected into the skin over a period of about 30 seconds. When the plunger had been pressed up to the limit allowed by the adjusted stop, the needle was held an instant and then slowly and carefully removed from the skin. In this manner no appreciable amount of dye leaked from the needle hole which was sealed by the mineral oil. After a moderate amount of experience had been gained by the use of the dissecting microscope it was possible to make the injections as well without it. However, in all other details the procedures described above were strictly adhered to throughout the experiments.

RESULTS

It was soon found that injections into the skin, in order to be comparable, must be made in similar areas dependent on the thickness of the fibrous layers of the dermis. In the guinea pig the three zones of the body, dorsal, lateral, and ventral, vary in thickness in about the proportion of 2:1.5:1 mm. and injections must be limited to one of these three zones. In fact, in a given normal animal the spread of dye in dorsal and ventral zones may vary by as much as 100 per cent. This difference measures a physiological variation in diffusion correlated with a difference in structure, that is, in thickness of the fibrous layer. In general we have limited the injections to either the dorsal or the ventral zones. When the

dorsal zone has been used, two injections have been made in the midline, spaced about 3 or 4 cm. apart; when the ventral zone has been used, we have made four injections in each of the four quadrants.

With the amount of dye used (0.025 cc.) a small bleb is formed at the site of injection from which the superficial lymphatics are filled at once. These lymphatics can be seen quite clearly through the mineral oil which makes the skin slightly translucent. From this superficial plexus the larger lymphatics of the subcutaneous plexus are filled almost immediately and the dye can then be seen extending in long streamers at this level. In the guinea pig the lymphatic trunks from the subcutaneous plexus penetrate the panniculus carnosus muscle only opposite the regional lymph nodes.

Besides this drainage of the injected dye into the lymphatics, there is a slow diffusion of dye from the bleb. In the area immediately surrounding the bleb, which usually measured originally about 2 mm. in diameter, the dye could then be seen to extravasate rapidly into the tissues so that by the end of 20 minutes the dark area measured about 5 or 6 mm. in diameter. From this period on the dye spread rather uniformly around the dark bleb and stained the skin a light blue, the limits of which could be seen and measured. It was found by repeated experiments that for purposes of comparison measurements taken at 1 hour, 4 hours, and 24 hours were sufficient to establish the rate of spread of the dye. The spread of dye in the skin of normal and tuberculous guinea pigs after 1 and 4 hours is illustrated in Figs. 1 to 4. When the dye has been injected for as long as 4 hours the limits of the spread in the normal animal become more difficult to ascertain and to measure on account of the hazy edge of the dye area, in contrast to the sharp, distinct border of the area in the skin of the tuberculous animal.

The measurements of the areas of the spread of dye in the different experiments are shown in Table I. They are classified according to whether the dye was injected into the dorsal or the ventral zones. In all instances there was a greater spread of dye in the thinner skin of the ventral zone. The first three groups of animals, namely, tuberculous guinea pigs in the allergic state, normal guinea pigs, and tuberculous in the hypoergic or anergic state, are arranged in the order of increasing spread of dye. The differences in the rate of spread were in all instances mathematically significant. In addition to the animals shown in the tables, ten normal and ten tuberculous guinea pigs have been studied more recently in the same manner with results showing the same ratio between tuberculous and normal. For measurements made after 24 hours, it will be noted in Table I that only eight measurements were made for the normal group. This was due to the difficulty in determining the outline of the area of the

primary injection, because the entire animal was stained, even to the foot pads, the tongue, and the conjunctivae. This extensive dis-

TABLE I

Areas of Spread of Dye in the Skin of Normal and Infected Guinea Pigs

	Ventral					
	1 hour		4 hours		24 hours	
Groups of animals	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
	sq. mm.		*sq. mm.*		*sq. mm.*	
Tuberculous, allergic state 40 injections 10 animals	192 ± 27.0	77.8	378 ± 17.0	158.0	1214 ± 46.0	435.0
Normal 32 injections 8 animals	218 ± 8.18	69.0	665 ± 27.0	228.0	*2102 ± 94.0	481.0
Advanced tuberculosis 12 injections 3 animals	461 ± 30.17	155.0	822 ± 37.12	190.6	1586 ± 97.14	498.9
Epizootic lymphadenitis 12 injections 3 animals	140 ± 4.96	25.5	477 ± 29.51	151.6	1359 ± 75.8	389.2
	Dorsal					
Tuberculous, allergic state 18 injections 9 animals	103 ± 4.7	29.4	166 ± 10.1	63.6	411 ± 21.5	135.5
Normal 18 injections 6 animals	121 ± 5.6	35.6	237 ± 11.4	71.7	678 ± 40.4	253.9
Advanced tuberculosis 6 injections 3 animals	181 ± 8.4	30.7	349 ± 22.2	80.8	700 ± 50.0	180.3
Epizootic lymphadenitis 6 injections 3 animals	116 ± 5.7	20.6	201 ± 8.7	31.5	502 ± 43.5	157.8

* Eight measurements.

tribution of the dye was due not only to a greater diffusion in the dermis but also to a far greater drainage of the dye into the lymphatics

and hence into the blood stream. On this account some of the primary areas could not be discriminated sharply enough to be measured.

The tuberculous animals whose records are shown in Table I have been divided into two groups according to the duration of the disease. In the first group the period after inoculation varied from 1 to 6 months and covered the time during which the animals showed a positive tuberculin reaction. An effort was made to see if the amount of restriction to spread of the dye was correlated with the degree of allergy but this was found not to be true, as demonstrated in Table

TABLE II

Areas of Spread of Dye in the Skin of Tuberculous Guinea Pigs with Reference to the Degree of Allergy

Guinea pig No.	Dorsal			Tested with 0.1 mg. tuberculo-protein MA-100	
	1 hour	4 hours	24 hours		
	sq. mm.	sq. mm.	sq. mm.		sq. mm.
*R 5042	163	300	629	++	237
R 5030	93	107	330	++	289
R 5031	106	173	380	++	374
Mean.........	122	193	446		297
R 5034	118	278	591	++++	1073
R 5044	107	160	449	++++	589
R 5038	72	106	266	++++	612
Mean.........	96	181	435		766

* These numbers represent serial numbers in the work of the laboratory over a term of years.

II, from which it is clear that there was no significant difference in rate of spread of dye between guinea pigs that showed two plus and four plus tuberculin reactions. The second group comprised those guinea pigs in which the loss of weight had been so great that it was estimated that resistance was failing rapidly. They were tested with the usual amount of tuberculo-protein, 0.1 mg. of MA-100, and were either entirely negative or gave a one plus reaction. When these animals were injected with the dye it spread faster than in the normal animal (Table I).

It is of interest to know whether such a restriction of spread of injected materials is present in any other infection besides tuberculosis. Three guinea pigs which had a spontaneous infection diagnosed as the epizootic lymphadenitis due to a Streptococcus infection, Lancefield type C, both by palpation of the enlarged lymph nodes and by four plus reactions with the bacterial extract prepared by Dr. Moen, were injected with dye. The results are shown in Table I. A comparison with the corresponding injections in normal guinea pigs shows a significant restriction of the dye but not as great as in tuberculous animals.

DISCUSSION

We have found that a vital dye injected into the skin of guinea pigs made allergic by two different infections, namely, tuberculosis and epizootic lymphadenitis, has a significantly slower rate of spread than in normal animals. The skin of animals in the hypoergic and anergic state of advanced tuberculosis, on the other hand, allows a significantly faster rate of spread of dye than the skin of normal guinea pigs.

It is clear from our experiments that at least two different processes are involved in this restriction to the spread of dye. They are a change in the rate of diffusion in the spaces of the connective tissues and a decrease in the permeability of the endothelium of the lymphatic capillaries. That there is a difference in the rate of diffusion can be seen by comparing the photographs of the spread of dye in the normal and in the tuberculous guinea pigs 4 hours after injection. In the normal skin the edge of the area showing dye is hazy, while in the tuberculous animal the corresponding area is as sharp as a knife's edge. The difference in drainage through the lymphatics into the blood stream is shown in the fact that when as much as 0.1 cc. of dye was injected, as in the four injections in the ventral region, the dye had spread throughout the normal animal in 24 hours enough to stain all the mucous membranes, whereas in the tuberculous animal not enough had drained into the blood vessels to be seen away from the place of the injection. This observation indicates one factor in the changed reactions of an animal in the allergic state has to do with permeability of endothelium. It is interesting to note that the work of Duran-Reynals (11) and of Hoffman and Duran-Reynals

(12) shows an involvement, but in reversed direction, of these same two factors in response to the injection of a spreading factor obtained from tissue extracts. They found that the injection of the spreading factor with dye was followed by an increased diffusion through the tissues, which could be demonstrated even in the skin which had been removed from the body. When they injected the spreading factor into the skin and the dye into the vessels, it was clear that the permeability of the vessels had increased because dye was concentrated into the area which had received the spreading factor. Thus this is the reverse of the situation which exists in the allergic state of tuberculous infection. These reactions of the skin in this allergic state are measured by a non-specific dye and hence cannot be related to an antigen-antibody reaction, though, as Opie (4) has shown, the antigen with which an animal has been sensitized is also restricted at the point of injection.

Our understanding of this complex phenomenon is far from satisfactory. There is a slight tendency toward edema when the dye is injected into the skin of the tuberculous animal. This is barely perceptible and can only be seen when closely observed and compared directly with the corresponding reaction in the normal animal. Parsons and McMaster (9) have shown that in a non-sensitized animal the spread of dye is increased while edema is forming. In the tuberculous animal, on the other hand, the development of edema does not counteract the tendency toward restriction. Thomas and Duran-Reynals (13) have shown that when the extent of the dermal reaction of a tuberculous animal to a test with a given amount of tuberculin is known, the addition of spreading factor to the same amount of protein markedly diminishes the reaction. When dye is added to the spreading factor, it can be noted that the spreading factor has created a condition which results in dilution and spread of the injected material. Guinea pigs in the advanced stages of tuberculosis, when they have become anergic to tuberculin, have lost the power of restricting the spread of dye in the skin and react rather as does the normal animal after receiving spreading factor.

From these observations it seems very probable that this phenomenon of restriction of spread of materials injected into the ski

in the allergic state may be one factor in the demonstration of the characteristic skin reactions. It may be that the increased irritability of the tissues and the changed permeability of the endothelium of the vessels have some share in concentrating the material injected, whether it is a specific, *i.e.*, antigen, or a non-specific irritant at the site of injection. Any concentration of an antigen around sensitized cells would enhance their reaction. The demonstration of a changed reaction toward diffusion in the tissues and a changed amount of drainage into the vessels by the use of a wholly non-specific dye material, combined with the fact that such changes can be induced, even though in the reverse direction, with tissue extracts (Duran-Reynals), indicates the possibility of the presence of some general as well as specific factors in the allergic state. It has thus been shown that infection with tuberculosis in guinea pigs induces changes which are manifested as an altering of the rate of diffusion in the connective tissues and of the rate of absorption through the vessels; these changes can be demonstrated not only by specific antigens but also by non-specific materials.

CONCLUSIONS

1. In the skin of the tuberculous guinea pig while it is allergic, the spread of a vital dye, pontamine sky blue, and the drainage of the dye into the vascular system take place much more slowly than in the normal animal.

2. In the skin of moribund tuberculous guinea pigs, animals no longer allergic, dye spreads more rapidly than in the normal animal.

3. The spread of dye is somewhat restricted in the skin of guinea pigs infected with a hemolytic Streptococcus. The animals were allergic.

4. The findings suggest that the dye method may disclose altered tissue conditions in the allergic state.

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EXPLANATION OF PLATE 10

FIG. 1. Spread of pontamine sky blue in skin of normal guinea pig, No. R 5324, 1 hour after injection of 0.025 cc.

FIG. 2. Spread of dye in skin of tuberculous guinea pig, No. R 5266, 1 hour after injection as in Fig. 1.

FIG. 3. Spread of dye in skin of normal guinea pig, No. R 5378, 4 hours after injection as in Fig. 1.

FIG. 4. Spread of dye in skin of tuberculous guinea pig, No. R 5266, 4 hours after injection as in Fig. 1.
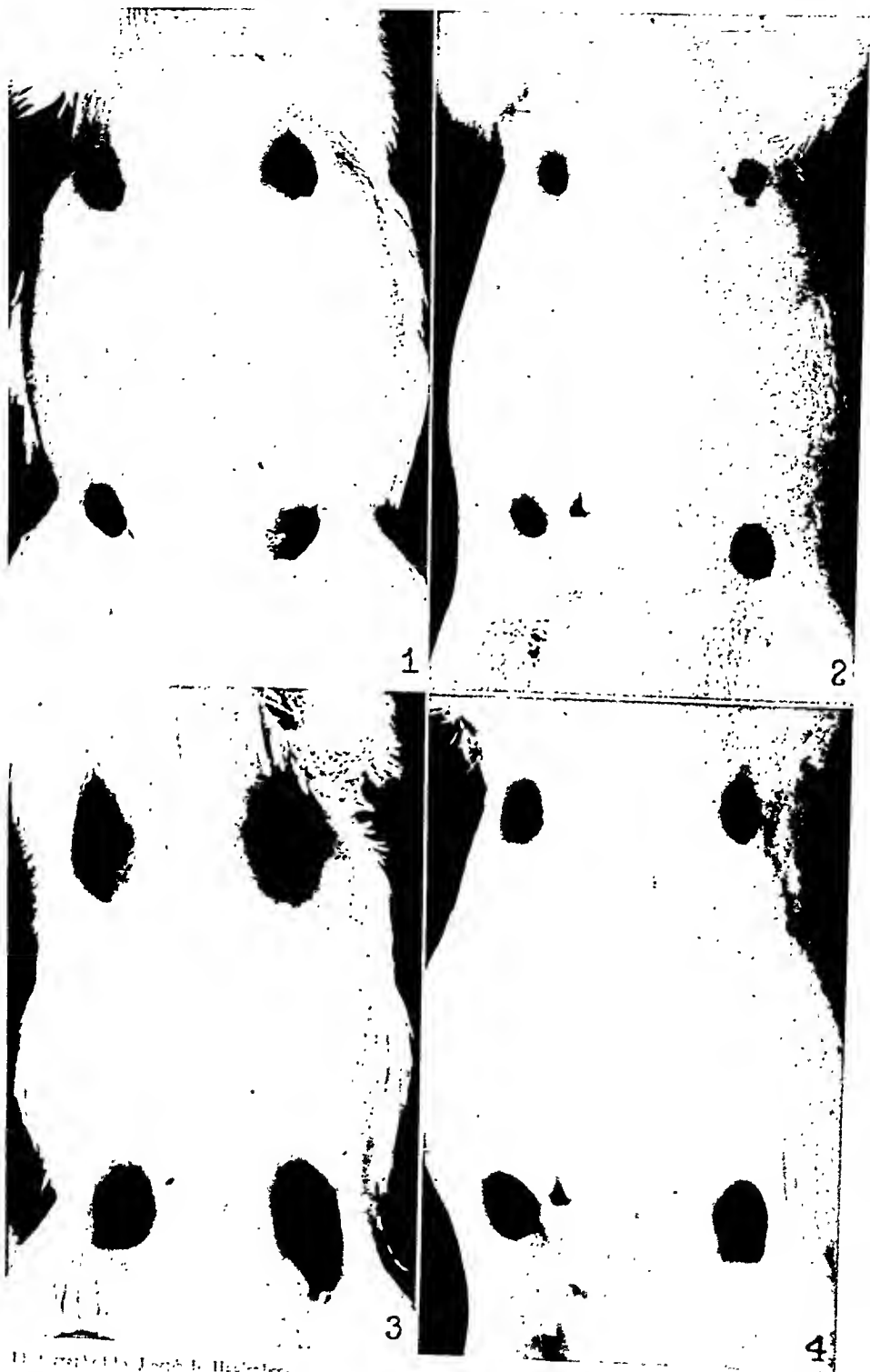
STUDIES ON THE ANTIGENIC STRUCTURE OF SOME MAMMALIAN SPERMATOZOA

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PLATE 11

Numerous studies of the antigenic composition of various bacteria have been made, but rarely has it been possible to identify certain antigens with definite morphological features of the cells. Exceptional cases are the members of the typhoid-paratyphoid group. Since the possibility of such a correlation of immunizing action with structural components depends somewhat on the degree of morphological differentiation it should be possible to carry out such analyses in the case of certain types of cells other than bacteria. Because of their extreme differentiation, involving almost complete localization of cytoplasmic and nuclear portions, mammalian spermatozoa are particularly suited to this type of analysis. The study of antigenic structure described in this paper was further facilitated by the discovery that the spermatozoa can be broken readily into heads and tails by sonic vibration, and that the two components can thereafter be separated by centrifugation. Several different antigenic materials have been demonstrated in the two fractions by subsequent serological procedures.

Materials and Methods

The preparation of spermatozoal suspensions as well as methods for preparation of antisera have been fully described (1).

Separation into Heads and Tails.—Separation of mammalian spermatozoa into heads and tails apparently has not been successful previously, although efforts to accomplish such cleavage have been made. Miescher (2) and, subsequently,

335

several others have separated heads from tails of piscine spermatozoa by the simple expedient of centrifuging in distilled water. The heavy heads broke off and stratified while the material of the tails stayed in the supernatant, but mammalian spermatozoa were not susceptible to this manipulation.

To fragment the gametes we have made use of the magnetostriction oscillator used by Chambers and Flosdorf (3) for the extraction of labile antigenic substances from *Streptococcus hemolyticus* and *Eberthella typhi*. The cell suspensions were exposed directly to vibrations at 9000 cycles per second of sufficient intensity to promote vigorous cavitation in the fluid. Temperature of the suspension was kept below 20°C. at all times by water cooling the inside of the nickel vibrating element. Descriptions of similar oscillators and the cooling arrangement have been published previously by Gaines (4) and by Chambers and Gaines (5).

After brief exposure to sonic cavitation it was found that practically all bovine spermatozoa in a heavy suspension were split into heads and tails. Final separation of the two components was accomplished by slow centrifuging. The heads settled down first and the majority of tails stayed in the supernatant liquid. By washing the first sediment four to six times an almost pure head suspension was obtained. After prolonged slow centrifuging of the first supernatant it was almost free of heads and subsequent high speed centrifugation for 30 minutes threw down the tails or at least the larger fragments of them. There was an obvious difference in the color of the sediments, the heads being milk white and the tails yellowish.

By the method described it was possible to obtain suspensions of either heads or tails of bull spermatozoa with 1 to 4 per cent of the unwanted part. The suspensions were counted in a Levy blood-counting chamber but the tail count cannot be considered exact since the tails usually were more or less fragmented. Only the larger fragments were counted. The supernatant liquid was not entirely clear after sedimentation of the tails and probably still contained small tail particles. Serologically the tails and the supernatant fluid behaved identically. It was therefore reasonable to assume that smaller amounts of antigen would be observed in the tail suspensions than would be found in the intact tails.

While the breaking of bull, dog, or rabbit spermatozoa was fairly complete after 7 minutes of vibration, 15 minutes were required for guinea pig cells, and 20 minutes for human. Final separation of guinea pig heads and tails was difficult as both came down at nearly the same rate during centrifuging. The best results which could be obtained showed about 12 to 15 per cent of the unwanted part. In human spermatozoa on the other hand it was very easy to get the pure head suspensions as the tails were entirely dispersed by the sonic

process.   In this case it was necessary to separate the heads from the seminal crystals which settled first.   Instead of a suspension of tails the supernatant liquid was used.   However, it was very difficult to obtain the large amounts of human spermatozoa necessary for these experiments and the same limitation hampered to a lesser degree the use of spermatozoa of dogs and rabbits.   Because of these difficulties bull spermatozoa and their parts were used most extensively and the male gametes of other species usually were employed only when confirmation was sought.

Smears prepared from the various suspensions and stained with methylene blue or Gram's stain revealed no differences in the surface of the heads before and after separation from the cells.   Only guinea pig spermatozoa lost their acrosoma.   Cleavage occurred uniformly at the junction of midpiece and head except in human spermatozoa. In the latter case a small part of the midpiece tended to remain with the head.   No further morphological changes were detectable upon dark field examination of fresh preparations.

Serological Tests.—Tests of antigenic activity were carried out by means of the complement fixation technique.   Equal volumes (0.25 ml.) of dilutions of antigen, antiserum, and complement were mixed together and incubated for 1 hour at 37°C.   Then 0.5 ml. of sensitized sheep red blood cells (2½ per cent) were added.   Two units of complement and two units of amboceptor were always employed.   Normal serum controls were included in all tests but never showed any reaction in the dilutions used and therefore are omitted from the tables.

Absorptions were performed in the following way.   Antiserum diluted one to ten was added to sedimented spermatozoa or to one of their parts, and the mixture was incubated for 30 minutes at 37°C.   After centrifuging, an equal volume of the corresponding suspension was added to the supernatant portion and this mixture was allowed to stand overnight in the refrigerator.   After centrifuging again at high speed for 1 hour the supernatant material was removed and incubated for 20 minutes at 56°C.[1]   The unabsorbed serum control was treated similarly except for the addition of saline solution in place of the spermatozoal suspension.   Final dilution of each serum was 1:20.

Slide agglutination and preparation of antibody solutions are described in the text.

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[1] The long centrifugation was necessary in order to get rid of smaller antigen-antibody complexes which of course would fix complement in the test as detectable by the saline control of the serum.   Even then we encountered occasionally a distinct fixation of complement in these controls.   This was due especially to the sera absorbed with homologous spermatozoa as in these cases a tremendous number of cells was required to remove all antibodies.

EXPERIMENTAL

1. Homologous Reaction.—In order to compare the reactions of the different fractions of spermatozoa with the homologous antisera the following experiment was performed. Five antigens were used, *i.e.*, 1, native bull spermatozoa; 2, vibrated bull spermatozoa; 3, heads of bull spermatozoa; 4, tails of bull spermatozoa; 5, the supernatant remnant of vibrated bull spermatozoa. All suspensions contained 10^8

TABLE I

Comparison between Bull Spermatozoa and Their Component Parts in the Homologous Reaction

Rabbit *versus* bull spermatozoal serum 32

Dilution of suspension	Suspension of bull spermatozoa or their parts 10^8 per ml.			Tails	Supernatant
	Whole cells	Sonically vibrated	Heads		
	0	0	0	0	0
1: 1	0	0	0	0	0
1: 3	0	0	wk	0	0
1: 5	0	0	c	0	0
1:10	0	0	c	tr	0
1:16	0	0	c	str	str
1:25	wk	wk	c	c	c
1:50	c	c	c	c	
1:80	c				
0					

0 = no hemolysis.
tr = trace of hemolysis.
wk = weak hemolysis.
str = strong hemolysis.
ac = almost complete hemolysis.
c = complete hemolysis.

cells or parts per milliliter and the supernatant solution was so diluted as to correspond to an equal concentration. Suspensions 1, 2, and 5 were derived from the same stock suspension of spermatozoa in order that the figures might be strictly comparable. However, under the conditions of preparation, 3 and 4 had to be counted separately. All the antigens were serially diluted and a constant volume of rabbit *versus* bull spermatozoal serum 32 in a dilution of 1:20 was added. The data obtained (Table I) showed that the heads contained less

antigen than the tails or the supernatant solution. There was regularly a slight increase of the antigenicity of the vibrated spermatozoa as compared with the native cells. This may be due to a greater dispersion of the antigen.

If instead of the different antigens the antiserum was serially diluted and a constant amount of the suspensions was added no striking dif-

TABLE II

Effect of Absorption with Whole Spermatozoa or Their Component Parts on the Reaction of a Homologous Antiserum with These Antigens

	Rabbit *versus* bull spermatozoal serum 34															
Dilution of serum	A Unabsorbed				B Absorbed with whole bull spermatozoa				C Absorbed with heads of bull spermatozoa				D Absorbed with tails of bull spermatozoa			
	Suspension of bull spermatozoa or their parts 5×10^7 per ml.															
	Whole cells	Heads	Tails	Saline	Whole cells	Heads	Tails	Saline	Whole cells	Heads	Tails	Saline	Whole cells	Heads	Tails	Saline
1:20	0	0	0	c	0	0	0	0	0	wk	0	c	0	0	0	0
1:35	0	0	0	c	wk	wk	wk	wk	0	ac	0	c	0	0	0	tr
1:50	0	0	0	c	c	ac	c	c	0	c	0	c	0	0	wk	str
1:100	0	0	0	c	c	c	c	c	0	c	wk	c	0	0	ac	c
1:160	0	0	0	c	c	c	c	c	wk	c	c	c	0	0	c	c
1:250	0	0	0	c	c	c	c	c	c	c	c	c	0	wk	c	c
1:500	0	0	0	c	c	c	c	c	c	c	c	c	str	ac	c	c
1:810	0	0	tr	c	c	c	c	c	c	c	c	c	c	c	c	c
1:1250	wk	wk	str	c	c	c	c	c	c	c	c	c	c	c	c	c

ference between the different parts of spermatozoa could be observed (compare Table II, column A).

By use of an absorption technique, however, it was possible to show that heads as well as tails possess antigens of their own (Table II). If a rabbit *versus* bull spermatozoal serum was absorbed with the whole bull spermatozoa no more complement fixation took place with either one of the parts save for the inhibition by the absorbed serum itself. After absorption with heads of bull spermatozoa there remained a distinct reaction with the whole cells and the tails, while

conversely, absorption with the tails left a marked reaction with the whole cells and heads.[2]

In order to identify further antigenic properties the different suspensions were submitted to heating at 100°C. for 20 minutes.

It is obvious from Table III that both heads and tails contained a heat-stable antigen. This substance is apparently identical in both parts of the cell as antibodies against it were absorbed by either heated heads or heated tails. Absorption with heads or tails in the native state, which left either the tail-specific or head-specific anti-

TABLE III

Demonstration of a Heat-Stable Antigen Common to Heads and Tails

Rabbit *versus* bull spermatozoal serum 32

Dilution of antiserum	Unabsorbed				Absorbed with 100°C. heads Suspension (5 × 10⁷ per ml.) of				Absorbed with 100°C. tails			
	Heads		Tails		Heads		Tails		Heads		Tails	
	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°
1:20	0	0	0	0	0	wk	0	str	0	ac	0	c
1:35	0	0	0	0	0	str	0	c	0	c	0	c
1:50	0	0	0	0	0	c	0	c	0	c	0	c
1:100	0	0	0	0	0	c	0	c	0	c	0	c
1:160	0	0	0	tr	0	c	0	c	0	c	0	c
1:250	0	0	0	ac	0	c	0	c	0	ac	0	c
1:500	0	tr	0	c	tr	str	str	c	wk	c	ac	c
1:810	0	ac	0	c	ac	c	ac	c	ac	c	c	c
1:1250	0	c	wk	c	c	c	c		c		c	c
1:2500	wk	c	str	c	c		c					

bodies, likewise removed the antibodies against the heat-stable substance. The absorbed sera did not react with heated heads or tails. The head-specific as well as tail-specific antigens are therefore heat-labile. These experiments were inadequate to determine the presence or absence of heat-labile factors common to both parts, but their existence is very likely since the remaining reactions after the absorption with the native parts are of relatively low titer in comparison

[2] The reaction with the supernatant remainder of vibrated spermatozoa equals that of the tails and was therefore left out of the table.

with the strength of the reaction after the absorption with the heated parts.

Such common factors appear to be responsible for somewhat indefinite results in the attempts to immunize rabbits with heads or tails of bull spermatozoa (Table IV).

While the head sera reacted to a much weaker degree with tails than they did with the homologous heads, the tail sera showed equally strong reactions with heads and tails. However, one must keep in mind that the suspensions of heads and tails never were entirely free from the other part. Since it seemed possible that the

TABLE IV

Comparison between Antisera against Whole Cells, Heads, and Tails

Dilution of antiserum	Antiserum against								
	Bull spermatozoa 34			Heads of bull spermatozoa 76			Tails of bull spermatozoa 78		
	Suspension of bull spermatozoa or their parts 5 × 10⁷ per ml.								
	Whole cells	Heads	Tails	Whole cells	Heads	Tails	Whole cells	Heads	Tails
1:50	0	0	0	0	0	0	0	0	0
1:100	0	0	0	0	0	str	0	0	0
1:160	0	0	0	0	0	c	0	0	0
1:250	0	0	0	0	0	c	0	0	0
1:500	0	0	0	tr	0	c	0	tr	wk
1:810	0	0	0	str	tr	c	0	str	str
1:1250	0	0	0	ac	ac	c	tr	c	ac
1:2500	str	tr	wk	c	c	c	ac	c	c

impurities of the suspensions used for the immunization might be responsible for the results with the tail sera we prepared other antisera with the supernatant remainder of vibrated spermatozoa. This material was found to react identically with the tail suspension. Because such preparations were free of heads we had reason to hope for antisera which would react dominantly with tails, but such was not the case since these sera reacted in the same way as the tail sera. The heat-stable common substance was found to be not entirely responsible for these cross-reactions.

The absorption experiments have shown that specific antigens exist in the heads and in the tails. We have also studied the effect of the

different antibodies by the slide agglutination reaction. For this purpose it was found to be advantageous to use motile spermatozoa, suspended in saline solution of pH 7.2 to 7.4. We were able to prepare the suspensions 2 to 3 hours after the bulls had been killed. Usually it was found necessary to incubate the suspension for a short time at 37°C. by which procedure the spermatozoa acquired their full activity. A drop of such a suspension was mixed with a drop of antiserum dilution on a slide and examined immediately under the microscope. Several different types of agglutination were thus observed in the different and variously absorbed sera.[3]

Absorption experiments were performed in the same way as shown in Table II. After the effect of the absorption had been established by the complement fixation test the sera were used for the agglutination reaction. When the unabsorbed serum was used fine strings of spermatozoa were formed immediately with attachment from head to head or tail to tail (Figs. 1 and 2).

Whether there occurred also a head to tail agglutination could not be surely established. When the spermatozoa were still living the heads might stick to these heads. When the motion ceased the heads were bent outward from the strings still moving and only other heads altered their position so that they often came into close proximity to the tails, but that seemed to be no antibody effect.

When the serum was absorbed with whole cells so that no reaction could be demonstrated in the preliminary titration no agglutination took place.

After the absorption of the serum with heads, only antibodies against the tails were left and these caused an immediate agglutination of the tails with all the heads at the outside of the agglutinate (Fig. 3). When the spermatozoal suspension was a little older but the cells still very actively motile, this type of agglutination reached a point where the tails were caught only at the ends so that around the small initial clumps a wheel-like formation occurred. The heads building

[3] In photographing these types we encountered several difficulties. The agglutinates are tridimensional and it is therefore impossible to get all cells into focus. When some of the agglutinates are most typical, the spermatozoa are still very motile; after the activity ceased or the cells were killed the cells bent or the clumps were altered.

the outer circle were not attached to each other as was quite obvious in the still living preparation. Pictures of this type could not be taken (see footnote 3), but Figs. 6 and 7 show such wheels with the addition of a head to head agglutination as produced by an anti-tail serum.

In this formation the "acidophile body" (6) at the end of the middle piece seemed to play an important rôle. In fresh spermatozoa this ring was mostly in place; when the suspensions aged a little the acidophile bodies slid down along the tails and small clumps of them as well as of whole spermatozoa seemed to form the centers of the wheels. This type was seen very occasionally in heterologous spermatozoal sera, never in normal rabbit serum.[4]

When the serum had been absorbed with tails, the heads clumped together forming long rows, the tails still striking vigorously (Fig. 4).

This type of agglutination has to be considered with reservations since a similar type may occur in normal rabbit serum or serum of several other species. However, a definite difference in the size of the agglutinates was observed, they being smaller, in normal serum than in antiserum. Furthermore the normally occurring agglutination was usually not found in dilutions higher than 1:10, while the antisera reacted up to 1:100 or greater dilution. In cases where the normal serum gave an agglutination the complement fixation test too showed slightly positive results.

Corresponding types of agglutination were observed with antisera obtained by injecting heads or tails of bull spermatozoa into rabbits. The head sera which reacted dominantly with heads in the complement fixation reaction (Table IV) showed correspondingly in higher dilution only the head type of agglutination. The tail sera fixed complement to the same degree with heads and tails (Table IV) but nevertheless they showed in the beginning of the agglutination the tail type. In low dilution a net-like formation was built immediately with all heads bent outward. This formation then shrank and the finer strings were disrupted, thereby clumps of different sizes were formed. Additional spermatozoa were caught at the end of the tail so that a rim of cells

[4] Sampson (7) saw a similar formation when spermatozoa of *Katharina tunicata* were exposed to homologous egg secretions or cytolized heterologous spermatozoa.

with the heads outside could be seen around the clumps (Figs. 5, 6, and 7). Often only a few cells or some of the acidophile bodies formed the center. After a while such clumps collided and a head to head combination resulted.

In order to confirm the results obtained by slide agglutination the same tests were carried out with antibody solutions. They were prepared by splitting off the antibodies from the antigen-antibody complexes at 56°C. according to the method described by Landsteiner and Miller (8) for the preparation of hemagglutinins. Heads or tails of bull spermatozoa were mixed with antiserum dilution and allowed to stand for 2 hours at room temperature. The fragments were sedimented and washed twice and resuspended in saline, incubated for 10 minutes at 56°C. with repeated shaking, then centrifugalized immediately while warm. The complement fixation reaction showed the presence of antibodies in the supernatant fluid. With antibodies split off from heads a slightly dominant reaction with heads was observed, while the antibodies obtained from tails showed the converse. The presence of common factors in the two parts has been pointed out before. The slide agglutination with these antibody solutions revealed types of agglutination corresponding to those given by the sera from which the antibodies were obtained.

2. *Heterologous Reaction.*—In a previous paper (1) it had been shown that antisera against spermatozoa possess a specific dominance for spermatozoa of the species from which the cells were obtained. The sera always reacted best with the homologous sperm cells, but more or less distinct cross-reactions occurred with heterologous spermatozoa.

To determine whether or not the cross-reactions were confined to only one of the parts, *i.e.* heads or tails, the following experiment was performed. Antisera prepared against spermatozoa of different species were allowed to interact with either heads or tails or supernatant solution (human), of bull, guinea pig, and human spermatozoa. Some of the results are given in Table V.

As shown in this experiment all antisera, no matter whether homologous or heterologous, reacted to about the same degree with the heads of bull and guinea pig spermatozoa when the antigen was increasingly diluted. Only the bull spermatozoal sera showed a weaker response

with guinea pig sperm heads. This is due to the lack of certain antibodies in these sera as will be explained later. A better homologous reaction was obtained with the heads of human spermatozoa.[5]

With the different tails as antigens we found a strong species-specific dominance although the degree of cross-reactivity of the different antisera varied. There were some heterologous antisera found, which did not react with tails at all.

TABLE V

Cross-Reactions of Heads and Tails of Spermatozoa of Different Species with Homologous and Heterologous Antisera

	Suspensions obtained from											
Suspensions of beads (10⁵ per ml.)	Bull				Guinea pig				Man			
	Antiserum (1:10) against spermatozoa of											
	Bull 35	Man	Rab-bit	Guinea pig	Bull 35	Man	Rab-bit	Guinea pig	Bull 35	Man	Rab-bit	Guinea pig
1:1	0	0	0	0	0	0	0	0	0	0	0	0
1:3	0	0	0	0	0	0	0	0	tr	0	0	0
1:5	0	0	0	0	tr	0	0	0	str	0	tr	tr
1:10	tr	wk	tr	wk	ac	0	0	0	c	0	str	wk
1:16	wk	ac	wk	ac	c	tr	wk	tr	c	tr	ac	str
Suspensions of tails (2 × 10⁵ per ml.)												
1:1	0	0	0	0	0	0	tr	0	tr	0	0	str
1:3	0	0	0	0	wk	0	wk	0	wk	0	wk	ac
1:5	0	0	tr	tr	ac	0	wk	0	str	0	str	c
1:10	0	wk	wk	wk	c	wk	c	0	ac	0	c	c
1:16	0	ac	ac	ac	c	c	c	0	c	0	c	c
1:25	0	c	c	c	c	c	c	0	c	0	c	c
1:50	tr	c	c	c	c	c	c	wk	c	tr	c	c

One might expect the heads to be definitely specific because of their biological function. The results obtained with the heads were therefore at first surprising. The heads do possess, however, a species antigen because the titer of an antiserum was always markedly higher with its homologous heads than with heterologous ones. Furthermore

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[5] Preparations of heads of human spermatozoa were shown to include fragments of tails in a higher percentage since the breaking does not always occur between head and midpiece.

a specific antigen in the heads could be shown simply by heating the heads for 20 minutes at 100°C. (Table VI).

The heating destroyed the heterologous reaction completely leaving a distinct homologous one. Spermatozoa or parts of them derived from other species also reacted only with their homologous antiserum after being submitted to heating. The heat-stable antigen is therefore species-specific.

When a heterologous antiserum was increasingly diluted and constant amounts of the different parts of the bull spermatozoa in suspension were added a striking difference in titer of the antiserum with the

TABLE VI

Influence of Heating on the Cross-Reactions

Dilution of suspensions	Antiserum (1:10) against spermatozoa of															
	Bull 33		Man		Rabbit		Guinea pig		Bull 33		Man		Rabbit		Guinea pig	
	Suspension of 10⁸ per ml. heads of bull spermatozoa								Suspension of 10⁸ per ml. tails of bull spermatozoa							
	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°	Native	100°
1:1	0	0	0	ac	0	c	0	ac	0	0	0	c	0	c	0	c
1:3	0	0	0	c	0	c	0	c	0	0	0	c	0	c	0	c
1:5	0	0	0	c	0	c	0	c	0	0	0	c	tr	c	tr	c
1:10	tr	tr	wk	c	tr	c	wk	c	0	tr	wk	c	wk	c	wk	c
1:16	wk	str	ac	c	wk	c	ac	c	0	str	ac	c	ac	c	ac	c
1:25	ac	c	c	c	ac	c	c	c	0	ac	c	c	c	c	c	c
1:50	c	c	c	c	c	c	c	c	wk	c	c	c	c	c	c	c

different parts was observed. Table VII illustrates an experiment of this kind.

There was a marked increase in titer after the spermatozoa had been vibrated, and this increase was apparently due to some change in the heads since neither tails nor supernatant gave such an increase. Corresponding results were obtained with spermatozoa of guinea pigs, dogs, and rabbits in the native and vibrated state. The only explanation for this phenomenon seems to be that a new antigen has come to the surface during sonic treatment. The cytological appearance of the vibrated cells has already been described, and it will be apparent that the only place where such a new surface could have appeared

was the site of breaking.   Only in the case of guinea pig spermatozoa was there evidence that the acrosoma had been torn away.

Other mechanical procedures such as washing several times, vigorous shaking, or grinding, also increased the titer of the heterologous antiserum when the damaged spermatozoa were used as antigen.   In such suspensions a considerable number of broken off heads were observed.

If a new antigen were responsible for this increase in titer, one should be able to demonstrate it by means of an absorption technique. Table VIII illustrates an experiment of this kind.

Rabbit *versus* human spermatozoal serum was absorbed with whole

TABLE VII

Influence of Sonic Vibration on the Titer of Heterologous Antisera

Dilution of antiserum	Rabbit *versus* human spermatozoal serum					Rabbit *versus* rabbit spermatozoal serum				
	Suspension of bull spermatozoa or their parts 5 × 10⁷ per ml.									
	Native	Sonically vibrated	Heads	Tails	Supernatant	Native	Sonically vibrated	Heads	Tails	Supernatant
1:20	0	0	0	0	0	0	0	0	tr	str
1:35	0	0	0	0	0	0	0	0	wk	ac
1:50	0	0	0	0	0	0	0	0	str	c
1:100	0	0	0	tr	0	0	0	0	c	c
1:160	tr	0	0	wk	tr	tr	0	0	c	c
1:250	wk	0	0	str	wk	wk	0	tr	c	c
1:500	ac	0	0	ac	c	c	wk	wk	c	c
1:810	c	0	wk	c	c	c	str	c	c	c
1:1250	c	tr	ac	c	c	c	c	c	c	c

bull spermatozoa or with heads or tails of bull spermatozoa.   The results show clearly that after absorption with the whole cells the antiserum still gave a strong reaction with the heads of bull spermatozoa though a weaker one than the unabsorbed serum.   Absorption with heads took out all antibodies.   Absorption with tails decreased the titer with the whole cells but left the reaction with the heads apparently unaffected.   This result constitutes definite evidence that a new antigen had come into action through the vibration.   Corresponding results were obtained when spermatozoa of other species were used as absorbents.   Other experiments show that absorption of an antiserum with whole dog spermatozoa for instance still leaves antibodies

against vibrated dog spermatozoa. After the sera were absorbed with heterologous cells or parts of them the reaction with homologous spermatozoa was either not detectably or only slightly decreased in titer.

The heterologous absorption experiment differed in some respect from the homologous one shown in Table II. It has to be noted that the homologous serum did not give a reaction with heads after absorp-

TABLE VIII

Effect of Absorption with Whole Spermatozoa or Their Component Parts on the Reaction of a Heterologous Antiserum with These Antigens

Rabbit versus human spermatozoal serum

Suspension of bull spermatozoa or their component parts 5 × 10⁷ per ml.

Dilution of serum	A Unabsorbed				B Absorbed with whole bull spermatozoa				C Absorbed with heads of bull spermatozoa				D Absorbed with tails of bull spermatozoa			
	Human sperm	Bull sperm	Heads	Tails	Human sperm	Bull sperm	Heads	Tails	Human sperm	Bull sperm	Heads	Tails	Human sperm	Bull sperm	Heads	Tails
1:20	0	0	0	0	0	wk	0	str	0	str	ac	str	0	0	0	tr
1:35	0	0	0	0	0	str	0	str	0	ac	c	ac	0	0	0	wk
1:50	0	0	0	tr	0	ac	0	ac	0	c	c	c	0	0	0	ac
1:100	0	tr	0	wk	0	c	0	c	0	c	c	c	0	wk	0	c
1:160	0	wk	0	str	0	c	tr	c	0	c	c	c	0	ac	0	c
1:250	0	ac	0	c	0	c	c	tr	0	c	c	c	0	c	0	c
1:500	0	c	wk	c	0	c	c	str	tr	c	c	c	0	c	wk	c
1:810	0	c	ac	c	0	c	c	c	ac	c	c	c	0	c	c	c
1:1250	0	c	c	c	0.	c	c	c					wk	c	c	c
1:2500	0	c	c	c	0	c										
1:4150	wk	c	c	c	str	c										

tion with the whole cells. Six different sera prepared against bull spermatozoa were absorbed with homologous whole cells but not one of them showed a reaction with heads of bull spermatozoa afterwards. Even when the serum was absorbed with the minimum amount of spermatozoa necessary for complete elimination of the reaction with the whole cells the reaction with the heads was gone. On the other hand when vibrated bull spermatozoa or heads of them were injected into rabbits antibodies were formed against this particular head antigen.

These antibodies were left in the serum in spite of absorption with the whole cells.

The results in cross-reactivity obtained so far are summarized in another form in Table IX which shows clearly that one may differentiate between three different cross-reacting antigens.

Antibodies against the first antigen (I) are absorbed by the whole cells and the heads but not by the tails; against the second one (II) they are absorbed only by the heads; and against the third (III) they are removed by all three, the whole cells, the heads and the tails.[6] Antigens I and III are on the surface of the cell, I is only on the surface of the heads, and it is not displaced by sonic vibration. Antigen II is believed to be covered by outside material in the whole cells

TABLE IX

Summary of the Results of Table VIII, Showing Three Cross-Reacting Antigens

| | Rabbit *versus* human spermatozoal serum | | | |
Antigens	Un-absorbed	Absorbed with whole bull spermatozoa	Absorbed with heads of bull spermatozoa	Absorbed with tails of bull spermatozoa
Whole bull spermatozoa........................	+	−	−	+
Heads of bull spermatozoa....................	+	+	−	+
Tails of bull spermatozoa.....................	+	−	−	−

and therefore does not react in the native sperm cell. Only after breaking off the heads does this antigen II come into action. Antigen III is probably reactive only on the surface of the tails.

This conception explains why the absorption with heads removed the antibodies against antigens I and II, but does not hold for the disappearance of antibodies against antigen III. The absorption of anti-III by the heads might be due to traces of antigen III on the heads or might be caused by the admixture of tails in the head suspension. As mentioned before 1 to 4 per cent of the unwanted part were always counted in the different suspensions, and the following con-

[6] Further evidence for a cross-reacting substance III lies in the fact that some heterologous antisera do not react at all with tails of bull spermatozoa though they give good reactions with whole cells and heads.

siderations might account for the loss of antibodies against antigen III: 4 to 5 × 10⁹ heads of bull spermatozoa were required to absorb 0.3 ml. of rabbit *versus* human spermatozoal serum, which means that 4×10^7 to 2×10^8 tails were present as impurities. On the other hand only 2 to 3×10^8 tails were necessary to remove from the same amount of this serum the entire cross-reaction with the tails. Therefore it is very likely that the impurity was responsible for the removal of anti-III by the head suspension.

To obtain further evidence of the cross-reacting antigens in bull spermatozoa it seemed desirable to remove if possible, the antigen I

TABLE X

Comparison between Specific and Cross-Reacting Antigens

Rabbit *versus* vibrated bull spermatozoal serum 75

Antigens	Unabsorbed	Absorbed with						
		Whole spermatozoa of			Heads of bull sperm	And whole dog sperm	Tails of bull sperm	And whole dog sperm
		Bull	Man	Dog				
Whole bull spermatozoa.....	+	−	+	+	+	+	+	+
Heads of bull sperm.........	+	−	+	++	−	+	++	−
Tails of bull sperm..........	+	−	+	++	±	+	++	±
Whole human spermatozoa..	+	+	−	±	±	−	+	−
Whole dog spermatozoa.....	+	+	−	+	+	−	+	+
Vibrated dog spermatozoa...	+	+	+					

from the surface of the heads without destroying antigen II. However even prolonged sonic vibration (1 hour instead of 7 minutes) was not successful. Digestion with various enzymes as pepsin, trypsin, and papain likewise proved to be unsuccessful.

So far it has been shown in the homologous reaction that in bull spermatozoa there is a head-specific as well as tail-specific heat-labile antigen besides a heat-stable species-specific substance common to heads and tails. In the heterologous reaction three different cross-reacting antigens could be established, two in the heads, one in the tails. It remained to show the relation between the homologous and heterologous reactions.

For this purpose we selected an antiserum prepared against vibrated bull spermatozoa since it gave the reaction with the inside material.

It is obvious from Table X that:

1. Absorption with whole cells of the bull left a reaction only against the inside material not only of homologous bull spermatozoa but also of vibrated heterologous spermatozoa. There is no reaction with homologous or heterologous whole cells.

2. Absorption with heterologous whole spermatozoa removed the reactions with the absorbent, but did not detectably alter the reactions with the various homologous antigens. There was also a reaction left with vibrated heterologous spermatozoa due to the inside material.

3. Absorption with heads of bull spermatozoa left a distinct reaction with homologous bull spermatozoa and tails and only a doubtful reaction with heterologous spermatozoa. When additionally absorbed with heterologous whole cells only the homologous tail-specific reaction was left.

4. Absorption with tails of bull spermatozoa removed the reaction with the absorbent but only slightly altered the other homologous and heterologous reactions. When additionally absorbed with heterologous whole cells the homologous head-specific reaction remained and also the reaction with the inside material of the heads of homologous and heterologous spermatozoa.

SUMMARY

1. A method has been described for separation of heads and tails of mammalian spermatozoa.

2. By means of absorption technique applied to homologous spermatozoal sera, head-specific and tail-specific antigens could be demonstrated. Both are heat-labile.

3. A heat-stable antigen was found to be common to both heads and tails. This substance is species-specific.

4. Antibodies against the head- and tail-specific antigens led to two different types of agglutination as shown by the slide method.

5. Using heterologous antisera against spermatozoa three different cross-reacting antigens could be observed, two in the heads, one in the tails.

6. One of the head-antigens is not active in the native cell; it comes

to action only after breaking the cell. Antibodies against this substance were not found in antisera against native bull spermatozoa but were formed when vibrated spermatozoa or heads were injected into rabbits.

7. The cross-reactions can be removed from an antiserum leaving the head- as well as the tail-specific reaction intact.

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EXPLANATION OF PLATE 11

FIG. 1. Net-like agglutination in the native whole cell antiserum. × 40.

FIG. 2. One of the strings under higher power. × 320.

FIG. 3. Tail type of agglutination. × 320.

FIG. 4. Head type of agglutination. × 320.

FIGS. 5 and 6. Agglutination of bull spermatozoa in a native antiserum against tails with clumps showing the rim of heads at the outside. Both figures × 80.

FIG. 7. The same under higher power. × 320.
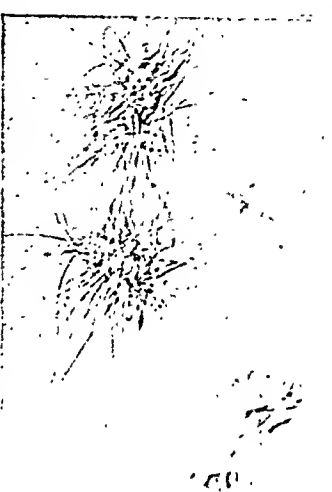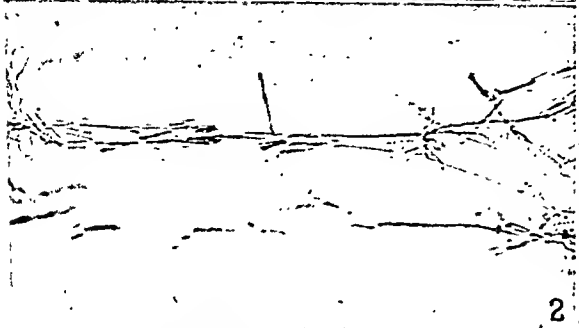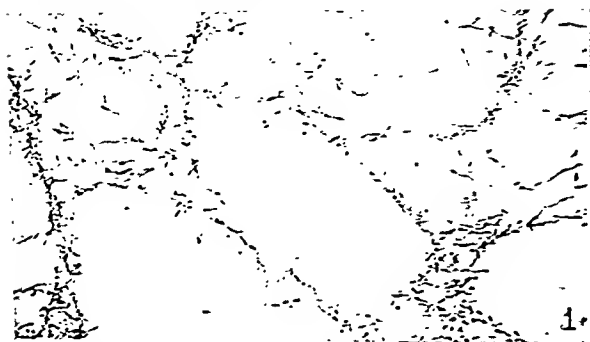
THE EFFECT OF THE PULSE UPON THE FORMATION AND FLOW OF LYMPH

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It is well known that massage and muscular movement increase the formation and flow of lymph (1–4). Undoubtedly pressure changes in the tissue are largely responsible. Nevertheless, the mechanism of lymph formation under such circumstances is not understood. Clearly the solution of the problem waits upon a better understanding of the factors influencing the extravascular transport of fluids through the tissues.

It has seemed wise in view of these findings to investigate the influence of the most natural of mechanical effects, that of the pulse wave, upon the formation and flow of lymph and the spread of substances through the tissues. The thought has suggested itself that the pulsations of vessels must serve, not only to facilitate the escape of substances from the blood, but to increase their spread through the tissues following their escape and, by an internal massage, as it were, to promote lymph formation and flow.

Previous Findings.—Earlier work has shown that changes in cutaneous lymph flow can be observed with the aid of vital dyes (4–6). On intradermal injection dye enters the superficial plexus of lymphatics through channels torn or ruptured by the injecting needle. As little as 0.01 cc. of a solution of pontamine sky blue or patent blue V will suffice for this purpose and renders them sharply visible at once. Later the dye can be seen, diluted and pale, draining away in the deeper channels appearing through the skin as colored streamers (4, 5). The streamers increase or decrease in length and intensity under conditions known to increase or decrease lymph flow respectively (4, 5). Intradermal injections of dye at the tip of the rabbit's ear (7) lead to the immediate appearance of color in the lymphatics there which extends rapidly to the base of the ear.

It has seemed possible to us that the effects of mechanical forces on the formation and flow of lymph could be determined with vital

dyes. To do this we have perfused the ears of rabbits with either a pulsating or a constant flow of blood at comparable pressure and have followed, in the manner to be described below, the movements of the dyes through the lymphatics. Before attempting the perfusion experiments, studies of the intralymphatic movement of the dye solutions were made in the ears of normal rabbits before and after subjecting them to conditions known to increase or decrease the flow of lymph respectively.

Preliminary Experiments.—At the tips of the ears of 25 normal rabbits 0.01 to 0.02 cc. of a solution of a vital dye, pontamine sky blue, the most indiffusible of many tested, was intradermally injected on the dorsal surface of the ear with a gauge 30 platinum-iridium needle, using as little pressure as possible. The dye, a 21.6 per cent aqueous solution isotonic with blood, diluted to 2 per cent with Locke's solution, has been employed by us before (8–10) in studies on the permeability of the lymphatic capillaries. As in our previous work on man (4–6), mice (8–10), and rabbits (7), the dye solution injected in this way entered the lymphatics through the superficial channels torn or ruptured by the injecting needle. As soon as it appeared in the channels the injection was stopped. As already shown (4–6), lymphatics injected in this way are not forcibly dilated or stretched, as in making fixed anatomical preparations of them. The deeply colored fluid is not driven into the entire channel to fill it, but instead diluted with lymph, it gradually progresses from the injection site toward the base of the ear. The movement, indicating lymph flow, was watched under a binocular microscope and with hand lenses. Tracings of the observed changes were made upon pieces of celluloid held over the ears.

In our first experiments this movement of the dye in the lymphatics was observed in the intact ear at rest, that is to say when lymph flow was least (1, 3, 4). Similar injections were then made in the opposite ears of the same animals under conditions attended by changes in lymph flow, that is to say following massage (1, 3, 4), after elevating the ear to a vertical position to promote lymph drainage (4), after irritation by paintings with xylol (9), or following the induction of hyperemia by heat (5, 9). For brevity only one of 25 experiments, typical of all, will be outlined, for the results were like those already reported in work on human skin (4, 5) and on the ear of the mouse (8, 9).

In the manner described, approximately 0.01 cc. of the 2 per cent dye solution was injected, within a few seconds, at the tip of one ear of a normal adult rabbit of the same size as those used in the perfusion experiments to be described later.

During the experiment the ear was held in a horizontal position. As soon as the dye solution appeared in the nearest lymphatic capillaries the injection was stopped. Natural sized tracings, showing the state of affairs immediately after the injection and the progress of the dye in the lymphatics at intervals thereafter, are shown in Text-figs. 1 *a* to 1 *g*. In this figure and in the others the irregular black areas represent the bleb of dye intradermally injected ½ cm. from the tip of the ear. The upper limits of the diagrams in Text-figs. 1 and 2 represent
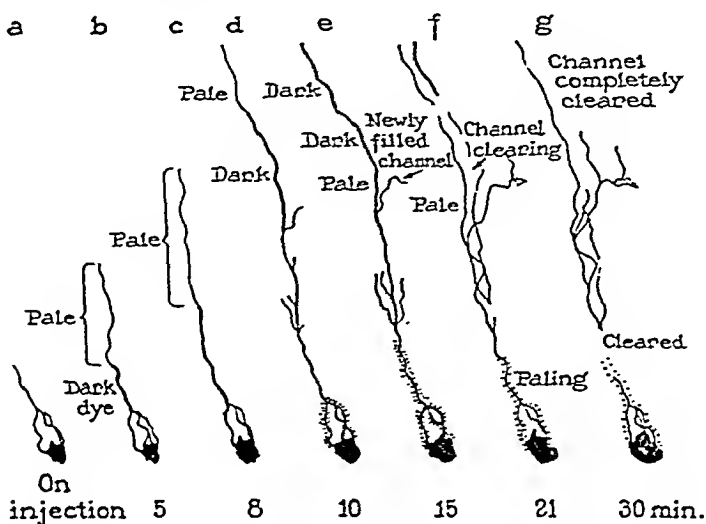
TEXT-FIG. 1. Tracings of the movement of color in the lymphatics of a normal quiet ear.

TEXT-FIGS. 1 to 8. In these text-figures the irregular black areas represent the bleb of dye intradermally injected ½ cm. from the tip of the ear. The upper limits of the diagrams in Text-figs. 1 and 2 represent the base of the ear. The upper limits of the remaining diagrams represent the cut edge of the amputated ears. These points were chosen so that the distance traversed by the dye would be approximately the same in all the experiments. In each text-figure the time interval, required by the dye to reach the base of the ear, has been underlined.

the base of the ear, as the tracings were made with the observer looking down upon the dorsal surface with the tip nearest to the body. The upper limits of the remaining diagrams represent the cut edge of the amputated ears. These points were chosen so that the distance traversed by the dye would be approximately the same in all the experiments. In each chart the time interval required for the dye to reach the base of the ear has been underlined. The first diagram, *a* in Text-fig. 1, shows the dye in the lymphatics immediately after the injection, which required but a few seconds. Within 5 minutes the column of colored fluid

in the lymphatic accompanying the central artery of the ear had reached the point shown in Text-fig. 1 *b*, dark where originally injected and pale in the extension.   3 minutes later a pale extension reached the point shown in Text-fig. 1 *c*.   Dye in this channel, in the outer and middle thirds of the ear then became darker, after the 8th minute, while at the same time a few small tributary channels appeared along the course of the large central one, bearing pale, diluted color as indicated in Text-fig. 1 *d*.   By the 10th minute pale colored fluid reached the base of the ear, while in the channel in the inner third of the ear, just behind the pale tip, lay the original dark column of fluid.   Lymphatics in the middle and outer thirds of the ear bore fluid which was not quite as dark as before.   Dye escape from the channels near the injection site had occurred secondarily.   We have
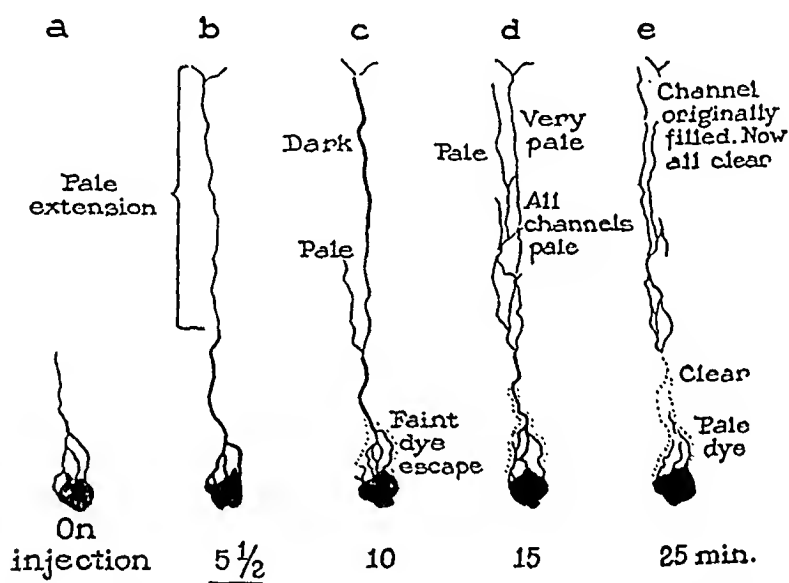
TEXT-FIG. 2. Tracings of the movement of color in the lymphatics of an hyperemic, quiet ear.

indicated this state of affairs in Text-fig. 1 *d* by the stippling.   5 minutes later, 15 minutes after the injection, paling of dye had taken place in the inner third of the ear just above a newly visible tributary channel, as though clear lymph were entering from tributaries themselves unseen.   At the same time the dark column of dye reached the ear base.   This is shown in Text-fig. 1 *e*.   By the 21st minute, Text-fig. 1 *f*, pale blue fluid reached the ear base in another newly visible channel. Many new channels carrying pale dye had appeared, and the color in the lymphatic originally filled had completely cleared in certain portions within the inner third of the ear.   Enough newly formed lymph had entered the lymphatic to sweep out all the colored fluid.   The main dye-containing channels close to the site of dye injection also had become much paler.   By the 30th minute that por-

ion of the lymphatic which first became visible and which lay in the inner third
of the ear, had become completely cleared, as also that segment lying close to the
dye injection, Text-fig. 1 g.

The course of events was typical of that in the majority of the
experiments done upon normal ears. However, large individual
differences occurred, due in part to variations in the size of the original
injections and to the extent to which dye ran toward the ear base
before the injection ceased. It must be stressed again that large
injections forcibly made can drive undiluted dye solution immediately
to the base of the ear. From such injections there can result no
indications of the state of lymph flow. In our experiments variations
in the rate of progress of dye in the lymphatics were dependent upon
the state of the circulation of the ear. For example in one instance,
in a flushed, warm ear, dye reached the base in 5 minutes, while in
cold ears with contracted blood vessels it required 16 to 20 minutes
in 3 instances, and even 40 minutes in one animal. In the remaining
21 experiments done on normal ears it required from 9 to 15 minutes.

In the perfusion experiments to be detailed below no such variations
as this occurred, for the conditions of flow and pressure of circulating
blood were controlled and much more uniform.

In each of the 25 experiments of the sort just described the animal's
other ear was subjected to procedures known to increase lymph flow,
that is to say the ears were warmed to produce hyperemia, or mas-
saged, or irritated by chemicals.

For example, an intradermal injection of about 0.01 cc. of the dye solution
was given at the tip of an ear after a 50 cc. centrifuge tube containing water at
55°C. had been held against the lower surface until a marked hyperemia appeared.
Following the injection, to avoid manipulation of the ear and to maintain hyper-
emia, light from a 100 watt electric bulb 20 cm. distant was allowed to shine on
the upper surface. A thermometer held on the ear surface registered 46°C. Dye
appearing in the lymphatics progressed toward the base much more rapidly than
in normal ears. As Text-figs. 2 a and b show, the pale and much diluted column
of color reached the base in only 5½ minutes. In the 12 other experiments of this
type, dye reached the base of the ear in 5 minutes on the average, with variations
from 2 minutes and 30 seconds to 9 minutes. In the test under consideration the
color in the central lymphatic became darker for a few minutes, but by the 10th
minute, Text-fig. 2 c, a definitely pale segment developed in that part of the
channel lying in the middle third of the ear. As no escape of dye had occurred

into the tissues the paling must have been brought about by colorless lymph coming into the channel from tributaries. The pale fluid in the middle portion of the lymphatic pushed the darkly colored material toward the ear base. At the same time that the paling increased, diluted dye appeared in a channel which had previously been invisible. By the 15th minute many new channels, one running almost to the base of the ear, had filled with pale blue fluid. The channel first filled with dye had become almost invisible in the inner third of the ear; for the dark blue fluid had been driven out of the organ. By the 25th minute this channel had completely cleared, Text-fig. 2 c, and so had those in the outer third of the ear. Dye escape, as shown by the stippling, was far less than in normal ears.

In all the 25 experiments in which the ears were subjected to one influence or another increasing the flow of lymph, as mentioned above, the rate of dye movement in the lymphatics was greatly accelerated. For example, during massage of the ears dye solution in the lymphatics reached the base in a few seconds. In ears with the tip held vertically above the base, color reached the latter in 3 to 5 minutes. In horizontal ears painted 2 to 5 minutes previously with xylol, dye reached the base, from the tip, in periods varying from $1\frac{1}{2}$ to $3\frac{1}{2}$ minutes, whereas in the ears at rest it required, as just reported, 9 to 15 minutes on the average, and never less than 5 minutes. In all these experiments, too, the color in the channels paled or cleared in far less time than in the tests done on the normal, resting ears. In view of these results we felt that the method could be used in the rabbit's ear, as it has been employed in human skin, to indicate changes in the rate of lymph flow. The clearance of color in the channels was indubitable evidence of a rapid formation of lymph and its transport in the lymphatics.

Such observations were next made in the ears of rabbits perfused with either a pulsatile or a constant flow of defibrinated rabbit blood under controlled pressure conditions.

Methods

A perfusion apparatus was devised by means of which warmed, aerated blood could be passed through organs under constant or pulsatile pressures and at alterable rates of flow. The operation of the device is best described by reference to Text-fig. 3.

Defibrinated blood, under pressure in a reservoir, R, was driven by pulsatile or by constant pressure, as will be detailed presently, through a one-way valve V_1 and thence through a warming chamber W into the ear E. Emerging from the
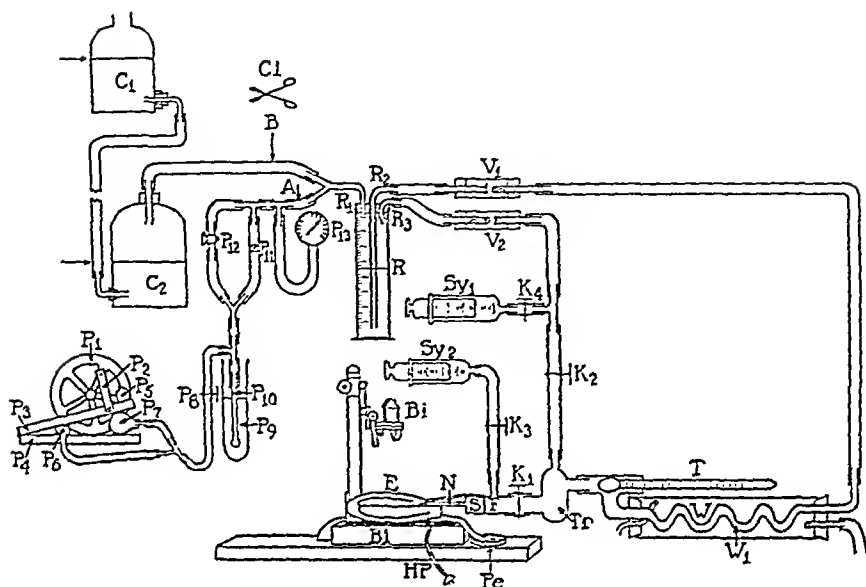
TEXT-FIG. 3. Diagram of the apparatus for perfusion of whole blood under constant or pulsatile pressure. See text for description.

C_1 and C_2, aspiration bottles; P_1, wheel which is connected through reducing gears and pulleys to 1/4 h.p. motor; P_2, connecting rod; P_3 and P_4, boards hinged at one end; P_5, 750 gm. weight; P_6, atomizer bulb; P_7, 250 cc. rubber bulb; P_8, screw clamp, to assist in control of volume of air; P_9, mercury in 25 mm. test tube; P_{10}, glass tubing with closely woven silk over lower end to prevent reflux of mercury; P_{11}, one-way ball valve from atomizer bulb; P_{12}, adjustable air valve for controlling "diastolic" pressure; P_{13}, Tycos blood pressure gage; Cl, clamp to be placed at A for constant pressure or B for pulsating pressure; R, blood reservoir; 100 cc. graduate closed with 3 hole rubber stopper, sealed with de Khotinsky's cement; R_1, air inlet; R_2, blood outlet; R_3, reflux tube for aeration of blood; V_1 and V_2, glass and rubber flap valves to control circulation of blood; W, water bath for warming blood; W_1, glass coils in water bath; T, thermometer; Tr, glass bubble trap and reflux connection; S, serum vial cap with 23 gauge platinum needle (without shank) pressed through it; E, rabbit's ear with needle tied into central artery; HP, electric connection to heating pad; Bl, wooden block; Pe, Petri dish to catch venous blood; Bi, binocular dissecting microscope on stand; K_1, screw clamp for control of blood flow; K_2, screw clamp for control of circulation of blood; K_3, spring clamp; K_4, spring clamp; Sy_1 and Sy_2, 20 cc. Luer syringes; N, needle— 23 gauge platinum-iridium; F, T tube.

veins it was collected, filtered through absorbent cotton moistened with Tyrode's solution, and returned to the apparatus. Large aspiration bottles, C_1 and C_2, partly filled with water and connected with the blood reservoir, as shown in the figure, imposed a constant pressure upon the perfusate. This could be increased or decreased by changing the level of the water in the bottles. Pulsatile perfusion pressures were generated, when desired, by the bellows-like action of two boards, P_3 and P_4, upon two rubber bulbs, P_6 and P_7. Compression of the bulbs forced air into a T tube, one limb of which led into the circuit of the perfusion apparatus while the other led through a tube to the bottom of a vessel partly filled with mercury, P_9. This device served as an overflow valve, and the mercury level could be changed at will, air bubbling out into the container when the air pressure rose above the desired level. The portion of the air column not bubbling through this valve was led through a Y tube into a double passage, one branch of which contained a one-way ball valve, P_{11}, through which it passed to exert the "systolic phase" of its pressure on the reservoir R. The other passage could be partially closed by the two-way stopcock, P_{12}. With the release of pressure on the bulbs, valve P_{11} closed while P_{12} was properly adjusted to allow just the right amount of air to leak backwards through it to maintain the desired "diastolic" pressure in the reservoir. A Tycos gage, P_{13}, in the circuit indicated the pressures and pressure changes in the reservoir.

The pressure in the reservoir forced blood through a one-way valve V_1 into a tube enclosed by a warming chamber for the adjustment of its temperature. Hot water flowed rapidly through the chamber and about the tube containing the blood. The flow of hot water was so regulated that the temperature of the blood passing the thermometer T was about 3°C. above that desired in the perfused organ. The warmed blood passed next into an air trap, Tr, and thence took one of two routes. Some, passing from the air trap through a short rubber connection, with a screw clamp, K_1, and then through a T tube, entered a 23 gauge platinum-iridium needle, capable of transmitting 18 cc. of blood per minute at a pressure of 141 mm. of mercury and at 38°C. The latter had been introduced into the artery of the organ to be perfused, in our experiments the central artery of the rabbit's ear.   -

The amount of blood entering the needle was controlled by the screw clamp, K_1. The remainder, passing out at the top of the air trap, returned through another one-way valve, V_2, to the reservoir R, flowing in through a flattened inlet tube R_3. This latter device directed the stream of blood toward the wall of the reservoir so that it spread out in a thin film on its passage down the glass walls of the container, while reoxidation occurred. The circulation and aeration of blood in the apparatus, as just described, was automatic only when pulsatile pressures were used. When constant pressures were used, blood was taken from the system at frequent intervals with the syringe, Sy_1, and returned to the reservoir by a slow reinjection after closing the clamp K_2, thus forcing it first through the valve V_2, then through the flattened inlet tube R_3 so that it ran down the inner surface of the reservoir and was reoxidized. The apparatus permitted the use of either constant

or pulsatile pressures when the clamp, C_1, was placed at either A or B, respectively, as shown in the diagram.

As already stated constant pressure was maintained in the blood reservoir R by the fluid in the bottle C_1 and varied by raising or lowering this bottle. The difference in the levels of the fluids in the two bottles C_1 and C_2 gave the measurement of the pressure. The pulsatile pressures were registered on a pressure gage P_{13}, and could be varied by changing the positions of the bulbs, P_6 and P_7, or by changing the level of the mercury, P_9 in the overflow valve. But the pressure at which blood entered the artery after leaving the needle tip was not known. To estimate it a series of preliminary tests were made. With the needle held horizontally, 30 cm. above the table, a constant flow of defibrinated blood was forced through at various known constant pressures. Beginning with a pressure of 160 mm. of mercury, we measured the distance that the stream of blood was thrown from the needle tip. The pressure was then lowered by 10 mm. of mercury and the distance spanned by the stream of blood again measured. This process was repeated, lowering the pressure by stages, 10 mm. of mercury at a time, until a pressure of only 30 mm. of mercury was exerted. Then, using the pulsating perfusion apparatus, different "systolic" and "diastolic" pressures were applied to the reservoir while we measured the distances that blood was thrown from the needle. For example, after it was found that the stream of blood was thrown 50 cm. from the needle tip by a constant pressure of 110 mm. of Hg, we connected the pulsation perfusion apparatus with the same needle and experimentally varied the pressure until blood was thrown equally far during "systole." The "systolic" pressure in the reservoir necessary to do this was found to register a pressure of about 145 mm. of Hg on the gage. Thereafter when a "systolic" pressure of 110 mm. Hg was desired at the needle tip, pressure was raised within the reservoir of the apparatus until the gage connected with it recorded 145 mm. of mercury. Similarly we noted the readings of the pressure gage when pressures were varied in the pulsation perfusion apparatus in such a way that the stream of blood emerging from the needle was thrown various distances corresponding to those determined when different constant pressures were applied. The "diastolic" pressures of the blood delivered at the needle tip were determined in the same way.

Perfusion Experiments.—Under ether anesthesia, the ears of adult albino rabbits were shaved. Through a small incision, directly over the central artery at the base of one ear, the vessel was isolated and ligated, leaving the ligature long. Now the ear was amputated at the base, weighed, placed on the warming pad (*HP* in the diagram), and at once the large-bored, 23 gauge needle was inserted into the artery and tied in place under a binocular microscope, *Bi* (Text-fig. 3). The needle, N, was fixed in a rubber stopper, S, which in turn fitted over a glass cannula connecting by rubber tubing with a syringe. Neither of the latter is shown in the diagram, but by means of them warmed Locke's solution was flushed gently through the ear as soon as the needle was in place. 20 or 30 cc. of the solution usually sufficed to clear the ear of blood, after which the stopper was taken from the cannula and fitted to the T tube, F. A syringe containing Locke's

solution was joined to the other end of the T tube as shown in the diagram at Sy_2. The long ends of the ligature on the artery were tied over the stopper S and the T tube tightly. The ear was placed horizontally with its under surface on a warming pad supported by gauze sponges so that the flat portion of the outer side could be observed and injected.

Except where specially mentioned, pooled, defibrinated blood, freshly taken from two or three rabbits, was used in all the experiments. Before each, cross agglutination tests were done by the method of Rous and Turner (11), using samples of the pooled blood and that of the animal from which the ear had come. No instances of agglutination were found. Early in our experiments it appeared that whole, defibrinated blood, however gently it was introduced into the ear after amputation, frequently caused an intense, transient spasm of the arterial tree. This phenomenon, well known to those who have perfused organs, was successfully reduced or even avoided by adding small amounts of blood to the warm Locke's solution after the first 20 or 30 cc. of the latter flushed out the animal's own blood already there. Following this, the screw clamp, K_1, was gradually opened, while 10 to 20 cc. of additional Locke's solution was gently injected at a uniform rate into the artery from the syringe, Sy_2. In this way the perfusate gradually became wholly blood, and the blood vessels of the ear relaxed until flow became apparently normal. The maneuver required 5 or 10 minutes, after which closure of the pinch cock, K_3, assured that only blood could reach the ear thereafter. The desired volume of flow was then attained by adjustment of pinch cock K_1. The blood emerging from the cut marginal veins, after its passage through the ear, was drained away by rubber dams, collected, filtered through cotton and gauze, and reinjected, as needed, into the perfusion apparatus through the syringe Sy_1. At varying intervals of 1 and 5 minutes during the experiments the temperature of the perfusate was recorded, together with the rate of blood flow, and adjustments were made if required.

The perfusion pressures employed differed in some experiments and were similar in others for reasons appearing in the following paper. As is well known, the volume of blood flow in the rabbit's ear varies greatly, the vessels being dilated at one time and constricted at another. For our experiments we determined to use those pressures which would yield sufficient flow of blood to the amputated organ to give it an appearance, under the binocular microscope, like that of a normal ear in which dye spots had been placed, when employing arbitrarily a "diastolic" pressure of 60 mm. of mercury. To obtain the desired blood flow it was found necessary in about half the pulsatile perfusions to employ a "systolic" pressure of 141 mm. of mercury. As it was further desired to maintain the constant pressure as high as the "systolic" pressures, we matched this figure in many experiments employing constant pressure. Of the experiments employing constant pressures, one was done at a pressure of 152 mm. of mercury, 9 at 141 mm., 4 at 131 mm., and 3 at 120 mm. of mercury. 6 of the perfusions with a pulsating flow of blood were done at pressures of 141/60 mm. of mercury, 5 at 131/60, 2 at 120/60, 1 at 115/60, 2 at 100/60, and 2 at 95/60. Some of these systolic pressures are higher than

those existing in the living rabbit (12–14), others probably equal to the normal pressures, or lower.

Regardless of the pressure differences, as will be seen below, lymph flow was faster in every ear perfused with a pulsatile flow of blood than in any of the ears perfused at constant pressure.

As soon as the proper flow of blood had become established, minute amounts of the dye solution were intradermally injected on the outer surface at the tip of the ear. The movement of dye in the lymphatics was then watched, and tracings made. Throughout the perfusions a lookout was kept for the development of edema. Under the binocular microscope, and with a fine needle, evidences of pitting on pressure were sought from time to time. The findings in all those instances showing edema will be considered separately.

When the last observations had been made in each experiment, 0.2 cc. of strong 21.6 per cent aqueous isotonic pontamine sky blue solution was injected through the stopper S of the perfusion apparatus (Text-fig. 3) so that the dye passed immediately through the needle into the circulating blood of the ear. The rate and character of its distribution about the lymphatics was noted, as was the rate of the blood flow through the ear. At the end of the experiment the ear was weighed again to determine the gain in fluid, if any, and sections were taken from various spots.

Save where especially mentioned, the data in this paper are based on perfusions with whole defibrinated rabbit's blood, the most satisfactory of the perfusates tested with a view to the maintenance of the tissues in an approximately normal state for an hour. These included Tyrode's solution, Locke's solution, Locke's solution containing 2 per cent gelatin, 7 per cent acacia, horse serum, whole or diluted with Locke's solution, beef serum, whole or diluted, beef plasma, whole or diluted with citrated Locke's solution, defibrinated beef blood, and heparinized or citrated rabbit's blood. Only with freshly defibrinated rabbit's blood were we able to perfuse the ear for an hour or more without the development of edema. In about half the experiments none occurred. The other perfusates containing plasma showed a strong tendency to form clots in the vessels, and hence their use had to be abandoned.

Findings

The data employed for comparisons of the rate of lymph flow were derived from experiments in which the perfusions with pulsating pressure involved a smaller volume of blood flow than those at constant pressure. The differences in the rate of flow are described in each instance. As more experiments were done at pressures of 141/60 and 141 mm. of mercury than at any other pressures the data charted have all been taken from such instances. Yet, as has been described, lymph formation and flow were much greater, in every instance, in

the ears perfused with a pulsatile pressure, that is to say with a pulsating current, regardless of pressure differences.

The Effect of Pulsatile Blood Flow upon the Formation and Flow of Lymph

In 17 experiments ears of rabbits were perfused at constant pressure, and in 18 at pulsatile pressure. The differences in the rate of lymph flow in the two types of experiment were so clear cut and constant that a brief description of one experiment of each kind will suffice. The injections were made, as already described, at various intervals after beginning the perfusions. The intervals had no effect upon the results. As in the experiments upon normal ears, the moment dye appeared in the superficial lymphatic capillaries, the injections were stopped. The progress of the dye along the draining lymphatic channels which accompany the central and marginal veins was timed and watched under the binocular microscope and traced on pieces of celluloid, as already described. The slight degree of pressure used to introduce the dye was the same in every instance. The differences to be described below cannot be ascribed to pressure changes.

The experiment in which the greatest movement occurred in an ear perfused at constant pressure will be compared with a typical experiment made with a pulsatile current of blood. As will be seen, the minute volume flow of the pulsating perfusate was far less and the "systolic" pressure far lower than the constant pressure of the former.

The right ear of a normal rabbit was amputated and perfused with defibrinated blood at a constant pressure of 141 mm. of mercury, in the manner already described. The ear weighed 12.2 gm. For 15 to 20 minutes before the dye injection of the lymphatics and for half an hour thereafter, the rate of blood flow averaged 4.01 cc. per minute or 0.33 cc. per gm. of ear. During the remainder of the experimental period, the flow increased to 4.65 cc. or 0.38 cc. per minute per gm. of ear. As usual, in these experiments approximately 0.01 cc. of dye solution was injected, requiring but a few seconds. By the time the injection was halted the color had already extended 5½ and 6 cm. in the central lymphatics (Text-fig. 4), much farther, that is to say, than in any of all the other experiments. This can be easily seen on comparison with the other diagrams. Secondary extension was slow nevertheless, the blue color extending less than 1 cm. in the

next 15 minutes (Text-fig. 4 *a*). After half an hour it had progressed 1 cm. further in one channel and about 1½ cm. in another (Text-fig. 4 *b*). The color in these extensions was pale and there was no escape of it into the surrounding tissue. Yet dye had escaped profusely from those portions of the channels originally filled by injection, as shown by the stippling in Text-fig. 4 *b*. In the next 16 minutes the movement was but 1 cm. more so that the colored fluid just reached the cut edge of the ear 46 minutes after making the injection. It had moved in all but 3½ cm. in three-quarters of an hour (Text-fig. 4 *c*). Flow in the second channel was slightly faster than that in the first, both vessels containing very pale blue fluid. Dye escape from the segment of the channel first filled became intense,
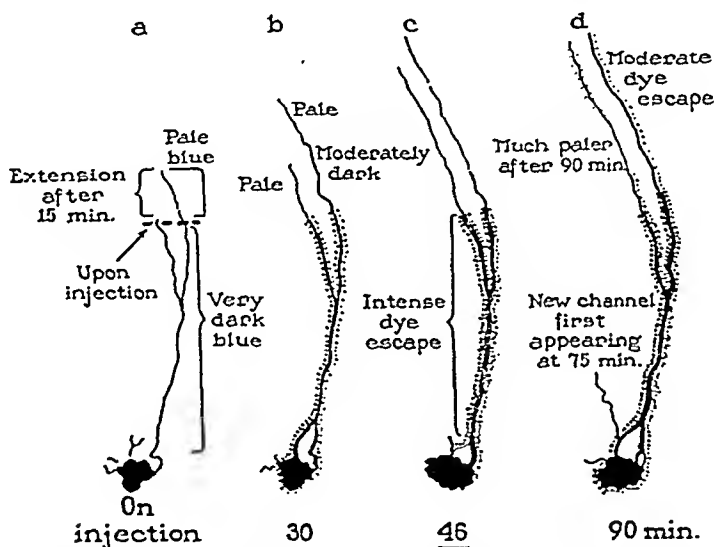
TEXT-FIG. 4. Tracings of the movement of color in the lymphatics of an amputated ear perfused at constant pressure.

leaving a great, broad band of dark blue about it. The dye in the lymphatics also remained dark blue and showed no evidence of becoming lighter during the experiment. From this it is evident that paling of the channels, as mentioned above, did not result from dye escape from them. After 1½ hours, a small segment of one of the channels containing pale blue fluid, as indicated in Text-fig. 4 *d*, became almost colorless, and a new channel appeared near the site of the injection carrying pale blue fluid. At the end of the experiment there was no detectable edema and the ear weighed only 0.2 gm. more than at the beginning. Although most of the experiments lasted an hour, this was the only one in which the color reached the cut edge of the ear in the lymphatics during a perfusion made with constant pressure, in an ear which did not become edematous.

Very different were the findings in ears perfused with pulsating blood. In a typical experiment now to be detailed, an ear weighing 13.2 gm. was perfused with a current of defibrinated blood pulsating 115 times a minute. Blood was discharged from the needle at "systolic" and "diastolic" pressures estimated to be 141 and 60 mm. of mercury respectively. It flowed at the rate of 1.54 cc. per minute for 15 minutes preceding the dye injection and at the same rate during the period of observation, that is to say, at about 0.12 cc. per minute per gm. of ear. The flow was, therefore, only one-third as fast as
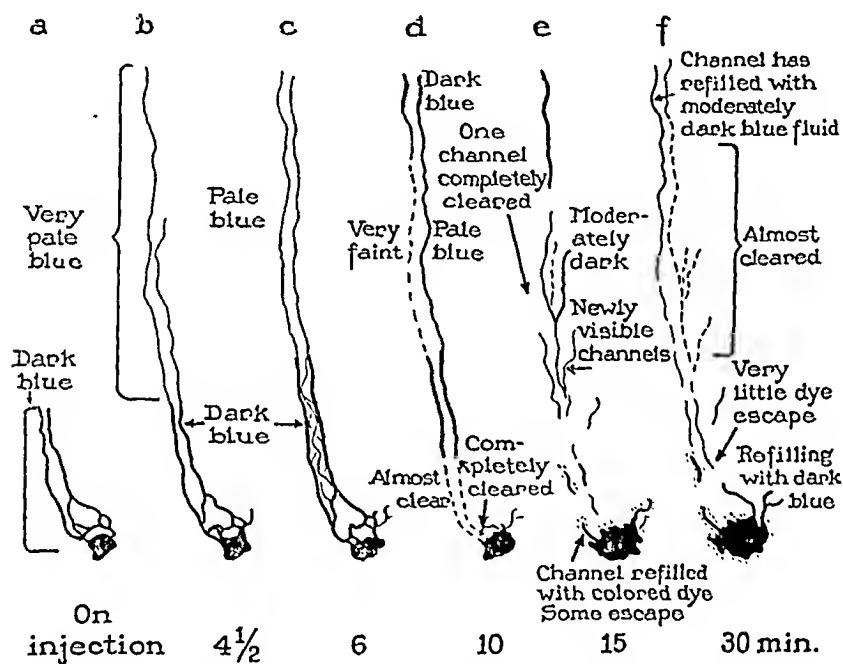
TEXT-FIG. 5. Tracings of the movement of color in the lymphatics of an amputated ear perfused at pulsating pressure.

that in the experiment just cited, and the "systolic" pressure just equal to the constant pressure. Despite this, lymph flow was 15 to 20 times quicker, as estimated by the rate at which color reached the base of the ear.

As in the experiment just cited, about 0.01 cc. of dye was injected intradermally near the tip of the ear in about 20 seconds. Text-fig. 5 a shows diagrammatically the dye in the lymphatics at the end of the injection. It is to be noted in this experiment that dye had not progressed as far down the ear upon injection as in

the case just cited. Nevertheless one streamer, pale and much diluted, reached the base of the ear, an additional distance of 5.5 cm., in 4½ minutes (Text-fig. 5 *b*), and a second one arrived there in 6 minutes (Text-fig. 5 *c*). At this time, too, many intercommunicating channels had filled with pale blue color between the two darkly colored lymphatics originally filled upon injection. The deep blue in these latter had extended toward the ear base. By the 10th minute the dye had become much paler in two of the lymphatics close to the injection site and had cleared completely in a third. In the meantime, some of the deeply colored material had been swept to the base of the ear, leaving the color very pale in the inner and middle thirds of the organ (Text-fig. 5 *d*). All the color had almost disappeared from one of these lymphatics, as indicated by the dotted lines. There were no signs of dye escape. Text-fig. 5 *e* shows that, during the next 15 minutes, the clearance of dye from the lymphatics near the tip of the ear had continued, the channels having become invisible in places. The more deeply colored fluid in the lymphatics continued toward the base of the ear in one channel, forced there by pale colored lymph now present in the lymphatics of the middle third of the ear. In this region pale colored fluid had entered many lymphatics hitherto invisible. A second channel in the inner third of the ear had now become invisible. There was still no evidence of dye escape from the channels except close to the injection site (stippling in Text-fig. 5 *e*). Within half an hour almost complete clearance in one of the channels in the mid third of the ear had occurred (Text-fig. 5 *f*), leaving a short segment of more deeply colored fluid near the cut edge of the ear. In the meantime, that channel near the cut edge which had been invisible 15 minutes before now contained deeply colored fluid. The observations gave clear evidence of rapid lymph flow.

The findings just described are in every way typical of those obtained during pulsating perfusion experiments. There was ample evidence of rapid lymph formation and flow, far exceeding that in the most favorable experiment upon an ear perfused at constant pressure. A comparison of typical experiments would have yielded even greater differences. Indeed no constant pressure perfusion resulted in as much, or as rapid, movement of color as the least movement that took place in the experiments with pulsatile perfusion. The effect of pulsation upon lymph flow is seen to better advantage in another type of experiment.

A rabbit's ear was perfused at a constant pressure of 141 mm. of mercury, the rate of flow being 2.7 cc. per minute. An intradermal injection of dye made in the usual manner colored two or three lymphatics blue, to the point shown in Text-fig. 6 *a*. For 12 to 14 minutes there was almost no movement of dye in the channels but much escaped through their walls as indicated by the stippling in

Text-fig. 6 *b*. This is characteristic of the constant pressure perfusions. For the constant pressure we then substituted "systolic" and "diastolic" pressures of approximately 141 and 60 mm. of mercury, respectively, exercising care to maintain the same rate of blood flow. Within 1½ minutes the color had advanced 4 cm. in one lymphatic, and 5 cm. in another (Text-fig. 6 *b*). The colored fluid in one channel reached the cut edge of the ear in less than 5 minutes, and in the second channel did so by the 6th minute (Text-fig. 6 *c*). As indicated in all the
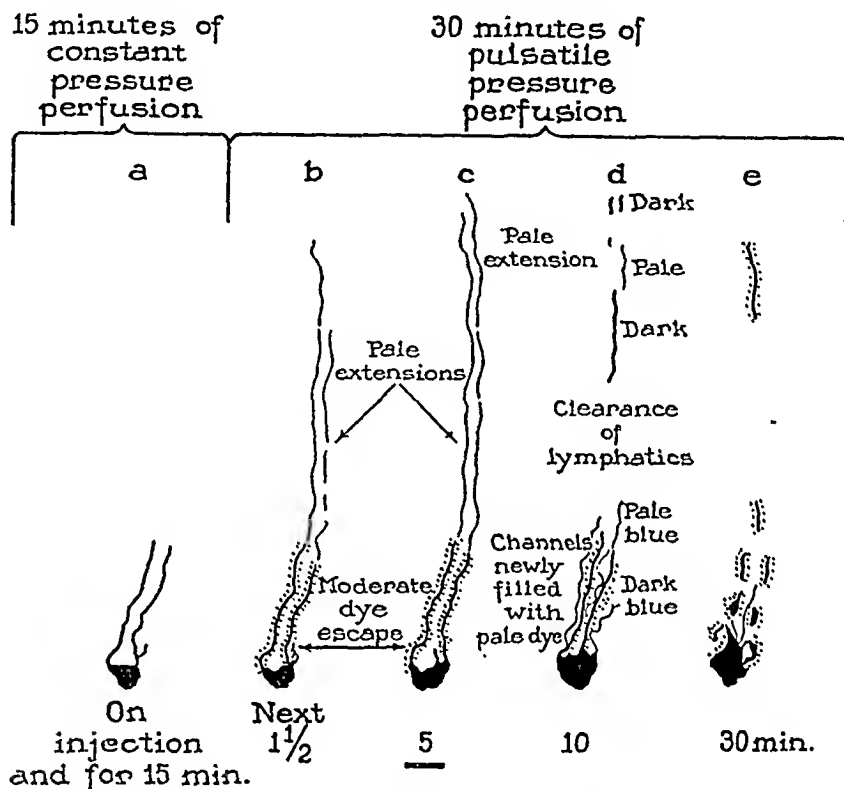
TEXT-FIG. 6. Tracings of the movement of color in the lymphatics of an amputated ear. (*a*) The lack of lymph flow while the perfusion was done for 15 minutes with constant pressure. (*b*) Active lymph flow 1½ minutes after changing to a pulsating pressure. (*c, d,* and *e*) Subsequent lymph flow and clearance of the lymphatics as the pulsatile current is continued.

figures, the colored extensions were far paler than those portions of the channels originally injected with dye. At the 10th minute after the shift to pulsatile flow, the color in the channels of the middle third of the ear had disappeared (Text-fig. 6 *d*). There was no evidence of dye escape from the lymphatics save in the regions close to the injection site, as indicated by the stippling.

The change from constant pressure to pulsating pressure in the perfusate brought about a great difference in lymph formation and

flow. All through the experiment, the rate of flow of the perfused blood remained approximately constant.

In all the ears which were perfused at pulsatile pressure, and which remained edema-free, the column of blue fluid in the lymphatics advanced rapidly toward the base, and reached it on the average in about 7 or 8 minutes, with variations between 3 and 11 minutes. By contrast, in only one of the experiments done at constant pressure in edema-free ears, did dye ever reach the base of the ear at all, and then only after three-quarters of an hour (Text-fig. 4). On the average, the color moved but 2 to 4 cm. in the half hour period usually allotted for the experiment. The average distance from the injection site to the cut edge of the ear was 8.5 cm.

In ears perfused with a pulsatile flow of blood, as also in normal and in hyperemic ears, the color of the draining lymphatics paled rapidly. Since the escape of dye from lymphatics to the tissues can readily be seen (6–8), it follows that the paling of the contents of the channels and their clearance of color was not due to the escape of dye through the lymphatics' walls but resulted from formation and flow of colorless lymph along the lymphatics. In all the ears perfused at constant pressure, the dark column of dye-stained fluid in the lymphatics advanced very slowly, and there was much more escape of dye from the channels into the tissue, yet the colored fluid did not become as pale within the lymphatics themselves as it did in the pulsatile perfusion experiments. In the latter there was less evidence of dye escape. It is of interest further that the paling of dye within the lymphatics and the subsequent disappearance of color began usually at the outer or middle third of the ear; the pale fluid entering the channels from tributaries, displaced the darker fluid, rapidly forcing it out at the proximal cut ends of the lymphatics. This latter phenomenon was never seen in ears perfused at constant pressure, save in one, during the formation of edema, as will be described below. On the average, in the experiments in which pulsatile perfusions were done, and no edema appeared, obvious paling of the lymphatics of the middle third of the ear began 8 to 15 minutes after dye reached that region; complete clearance of portions of the channels (shown in Text-figs. 5 and 6) began to occur in about 15 to 20 minutes. There was almost no clearance of the lymphatics when constant flow was used.

The differences described were found consistent and they constitute clear evidence that the presence of the pulse is of primary importance for the formation of lymph and, by corollary, in the maintenance of lymph flow. In ears perfused at constant pressure, there is almost no lymph flow.

In a few experiments, tracings were made of the movement of colored fluid in superficial lymphatic capillaries draining micro-puncture wounds which had been filled with dye solution by a method to be described in the following paper. When the ear was perfused at pulsating pressure, colored streamers extended 2 to 5 cm. from many of these dye spots. Similar phenomena were never seen in ears perfused at constant pressure.

The Effect upon Lymph Flow of a Developing Edema in Tissues Perfused with Pulsatile and Non-Pulsatile Current

The phenomena just described were noted in ears free from demonstrable edema; but the occurrence of edema in many instances offered an opportunity to study its effects upon the formation and flow of lymph. Edema developed in 8 out of 17 experiments, during perfusion at constant pressure, and in 6 out of 18 during pulsatile perfusion. In these cases edema was already demonstrable in the ear and was increasing when the dye was introduced into the lymphatics. The rate of dye movement within the lymphatics also increased while this was happening and this effect was much more evident when pulsating currents were used.

In the ears becoming edematous while perfused with a pulsating current of blood, dye reached the cut edge of the base of the ear within $2\frac{1}{2}$ minutes, on the average, with variations between $1\frac{1}{2}$ and 7 minutes. The dye movement was more rapid than that occurring within the lymphatics of non-edematous ears perfused in the same way, and much quicker than in normal resting ears. In the ears becoming edematous a paling of the colored contents of the lymphatics and complete clearance of portions of them took place with great rapidity, that is to say within $5\frac{1}{2}$ to 11 minutes, and 7 to 25 minutes respectively. The clear segments enlarged and extended rapidly toward the base of the ear, forcing the darkly colored fluid out of the cut lymphatics there.

Tracings of a typical experiment are shown in Text-figs. 7 *a* to 7 *d*. The characteristic progress of the colored streamers is obvious and may be compared with that in Text-fig. 5. As in most of the experiments done at pulsatile pressure, the rate of flow of the perfused blood per gram of ear was slower than in the perfusions done at constant pressure. In this instance it varied between 2.0 and 1.5 cc. per minute or 0.2 and 0.15 cc. per gm. of ear per minute respectively, for the ear weighed about 10.0 gm. "Systolic" and "diastolic" pressures of 141 and 60 mm. of mercury were used. The usual small intradermal injection of dye solution at the tip of the ear filled the channels for 2.5 to 3.0 cm. Pale streamers of dye reached the cut edge of the ear with great speed, in 2½ and 3 minutes (Text-fig. 7 *b*).
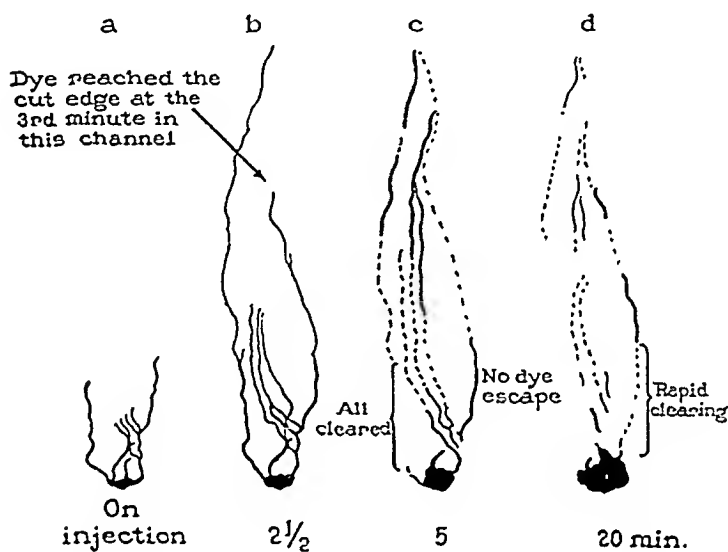
TEXT-FIG. 7. Tracings of the movement of color in the lymphatics of an amputated ear which became edematous while perfused at pulsating pressure.

By the 5th minute a large segment of one channel near the base showed complete clearance of dye-colored fluid. The lymphatics in the middle third of the ear had become very pale, as indicated in the figure by the dotted lines, while the dark colored fluid had been displaced toward the base of the ear and could be seen draining from the lymphatics at their cut ends. There was almost no dye escape into the tissues. By the 20th minute clearance of the lymphatics was marked, and there was still practically no dye escape into the tissues.

In 3 of the 8 constant pressure perfusions in which edema of the ears developed, the dye in the lymphatics reached the cut end of the

channels, an average distance of 8.5 cm., in 22, 26, and 30 minutes, respectively, at an average time of 26 minutes. In the remaining experiments it progressed only 4 or 5 cm. toward the cut base of the ear, moving that is to say almost as slowly as in the non-edematous ears which were also perfused at a non-pulsatile pressure. It is to be recalled, however, that in only one of the latter instances did dye reach the base and then it required 46 minutes to do so. Furthermore, in the absence of edema, the dye solutions diffused from the lymphatics into the tissue. Paling of the colored contents of the lymphatics did not occur, save in one instance (Text-fig. 4), and then it required $1\frac{1}{2}$ hours. In the 8 instances in which edema appeared, there was a slow but definite paling of the channels in 3 instances, but it required 30 minutes to 1 hour to become definite: it was in no way comparable with that seen in the experiments at pulsating pressures or in the intact, hyperemic, or normal ear.

For a direct comparison of the most marked findings obtained during perfusion at constant pressure while edema was developing, with typical, average findings in the pulsatile perfusion experiments involving edema, or in the tests on normal ears, we have reproduced the tracings from the constant pressure experiment in which color moved the fastest and paling of a lymph channel occurred soonest (Text-fig. 8). The pressure in this instance was 141 mm. of mercury. The rate of blood flow through the ear was faster than in any of the experiments done with pulsatile flow,—0.33 and 0.4 cc. per gm. of tissue per minute for the first and second half hour periods of the experiment respectively. The flow was therefore approximately double that of the experiment just described and charted in Text-fig. 7.

As Text-figs. 8 a and 8 b indicate, there was no movement of the colored solution in the lymphatics during the first 5 minutes following the injection, which required the usual few seconds. There was little progress of dye in the lymphatics in the next 3 minutes (Text-fig. 8 c), but some escape of dye, as shown by the stippling. At the 22nd minute pale extensions of color in two channels had reached the cut edge of the ear (Text-fig. 8 d). Already there had been much dye escape from the portions of these lymphatics which first became colored during the injection, and now they had become slightly paler, although still well colored. This fact cannot be shown in the diagrams. At about the 40th minute the paling increased and there was partial clearance of these channels in the regions

indicated by the dotted lines in Text-fig. 8 *e.* By the 45th minute the clearance was definite. Several new channels had appeared containing pale blue fluid, one of them reaching the cut edge of the ear, and there was some clearance close to the injection site.

The findings demonstrate that lymph formation and flow is only slightly greater than that observed in the constant pressure perfusions in which there was no edema. In spite of the presence of edema and excess fluid in the tissues, lymph flow is not as rapid as
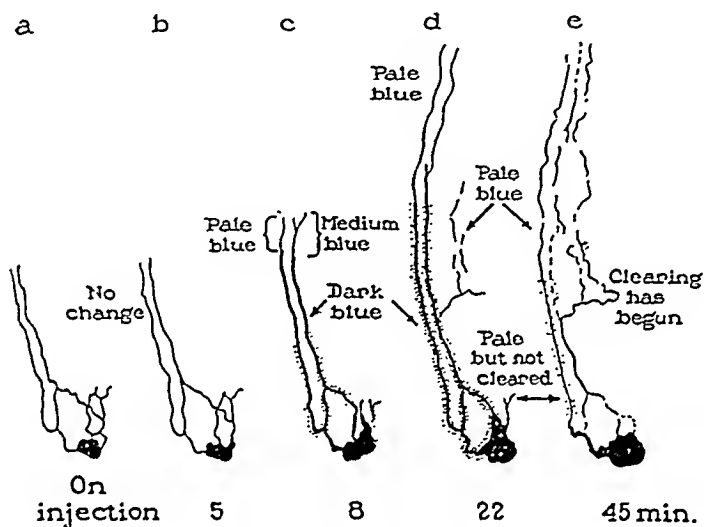
TEXT-FIG. 8. Tracings of the movement of color in the lymphatics of an amputated ear which became edematous while perfused at constant pressure.

in normal ears, or in non-edematous ears perfused by pulsating blood, and not nearly as rapid as in edematous ears perfused with pulsating blood.

It is to be stressed here that we have been able to compare the changes in lymph flow only during the formation of edema. Since it increased progressively as long as the perfusions were continued we were unable to ascertain the state of lymph flow when edema is fully formed or receding.

DISCUSSION

The method employed showed itself sensitive enough for the demonstration of changes in lymph flow in the rabbit's ear under conditions of hyperemia, change in position, and irritation. In all these states there was readily demonstrable the increased lymph flow which is known to take place (1–4). Color moved along the lymphatics more quickly under these conditions and the channels cleared of dye more rapidly.

One must infer from our experiences that there was little or no formation of lymph in the non-edematous ears perfused at constant pressure when edema failed to occur. Under these latter circumstances, however, with excess fluid in the tissues, there was evidence of a slow fluid movement in the lymphatics. It was far less than in normal ears or in ears perfused at pulsatile pressure, especially those which became edematous. Always during perfusion at pulsatile pressure, lymph flow was active whether or not edema formed.

It is well known too that tissues perfused with a pulsatile flow do far better than those perfused without this advantage. Our experiments disclose one reason why this is so. What happens through the agency of the pulse that has so great an effect to increase the formation and flow of lymph? In many of the pulsatile perfusion experiments blood flow was but 1/4 or even but 1/6 that of the non-pulsatile experiments, yet in the latter far less lymph was formed. In not a few of the experiments with constant pressure the ears became edematous, demonstrating that large amounts of fluid had escaped from the vessels. Nevertheless lymph flow was not promoted to any great degree. Obviously our findings must be ascribed to the mechanical effects of the pulse. The great flow of lymph in ears that were becoming edematous under pulsatile pressure, as compared with the slight flow when pressure was constant, may very well be ascribed to the massaging effect of the pulse. The influence of this last to increase the formation of lymph is more difficult to understand; and consideration of the problem presented will be deferred until further evidence has been set forth in later papers.

One further point requires mention. In previous work (5) it was shown that in the edematous skin of resting horizontal legs of aged patients suffering from cardiac edema there was no flow of lymph,

whereas in the edematous legs of youthful patients with nephritis, lymph flow was excessive. The presumptive effect in the cardiac patients of increased venous pressure obstructing lymph flow was ruled out, by the demonstration (5) that normal lymph flow took place in the non-edematous arms of these patients, when the arms and legs were at the same level in relation to the point of entrance of the thoracic duct into the great veins of the neck. No explanation for the excessive lymph flow in the nephritic patients was forthcoming.

The present experiments cast some light upon the state of affairs. In the rabbit ears perfused with pulsatile current, and in which edema occurred, lymph flow was excessive. In edematous ears perfused without pulsation it was very slight. The state of affairs in the ears under the circumstances first mentioned was in some ways comparable to that of the skin of the youthful nephritic patients in whom the action of the pulse was excellent (5), whereas that under the second set of circumstances may be likened to the condition in the cardiac patients in whom the pulse was not so good. In these latter patients, of course, the flow of blood was often reduced as well.

SUMMARY

The ears of rabbits were perfused with defibrinated rabbit's blood in such a way that pulsation could be imparted to the perfusate or withheld from it at will. In the absence of pulsation there was almost no lymph flow, whereas when it was present lymph flow was rapid despite the fact that the "systolic" pressure of the perfusate never exceeded the constant pressure in the non-pulsatile instances and the volume flow was far less.

Non-pulsatile perfusion led to a slight flow of lymph in ears that were becoming edematous, whereas when it was pulsatile the lymph flow was enormous.

The pulse exercises an influence to move fluid into the lymphatics and along them.

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THE EFFECT OF THE PULSE ON THE SPREAD OF SUBSTANCES THROUGH TISSUES

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Pulsation of the blood vessels in the ear of the rabbit increases the formation and the flow of lymph, as the preceding paper (1) has shown. In the present work we report the results of an investigation of the effect of the pulsation of blood vessels upon the interstitial spread of vital dyes through the skin of intact ears of normal rabbits, and through that of amputated ears perfused with defibrinated rabbit's blood.

Methods

To measure the spread of materials through the tissues of the rabbit's ear isotonic solutions of two vital dyes, pontamine sky blue and patent blue V, were employed. The former, a relatively indiffusible dye, was made up in 2 and in 21.6 per cent aqueous solutions isotonic with blood. The patent blue V, a highly diffusible dye, was employed in 11 per cent aqueous solutions, also isotonic with blood. Both dyes have been used in our previous studies upon the physiology of the lymphatics (2–7). The solutions were introduced without pressure into the tissues of the rabbit's ear in the following manner. With a dissecting needle, ground as finely as possible and under a binocular microscope, minute punctures were made through the epidermis into the subpapillary layer of the corium. The tip of a micropipette, 1/10 mm. in diameter and filled with dye solution, was gently touched to the tissue in the puncture wound. The dye solution filled the cavity by capillarity and without pressure, about 1/20 c. mm. entering in this way, as we have found. From this reservoir the dye spread slowly through the skin just beneath the epidermis. As result, there appeared a colored spot almost circular in form and from 0.8 mm. to 3.3 mm. in diameter. The spreading dye lay in a shallow layer with a smooth marginal outline which remained well defined, as a rule, for more than an hour. A gradual paling took place at the periphery as the dye spread through the tissues, although the margin remained smooth. Gradually too the whole spot of dye became pale. The spots were measured, as will be described below, only while the margins of color were well defined. To indicate that these small pools of dye solution lay interstitially under no pressure and that they were not forcibly injected to form a bleb, they will be termed dye maculae,

for they resembled in shape the common pigment maculae of the skin. The dye maculae could not be made uniform in size, a circumstance that raised certain problems to be discussed below.

In many experiments the small micropuncture wounds ruptured lymphatic capillaries, which the dye solution promptly entered. In other instances, especially during the formation of edema, dye solution oozed out from the maculae and spread upon the surface of the skin. No attempts were made to measure the spread of the maculae under either of these conditions.

Within less than a minute after the dye came in contact with the tissues the outlines of the resulting dye spots were drawn by means of a camera lucida attached to a binocular dissecting microscope. Later the areas of the drawings were determined by a planimeter. Similar measurements and tracings were repeated after half an hour and again after the lapse of an hour. For the sake of simplicity the initial areas are expressed in planimetric units. The relationship of these units to the actual size of the spots will appear below. The spread of the dye has been expressed in terms of the increasing areas of the maculae. For example, if the final area of a dye macula, expressed in planimetric units, was $2\frac{1}{2}$ times as great as its initial area, the spread was called 2.5.

Perfusion Experiments.—To determine the effect of the pulse upon the spread of dye through tissues we perfused amputated ears of rabbits with a pulsatile or non-pulsatile flow of defibrinated rabbit's blood, using for the purpose a technique and apparatus described in the preceding paper (1). The dye solutions were introduced into the perfused ears and the resulting spread measured as just described. All the rabbits used for this work were of about 2000 gm. body weight.

The preceding paper has shown that lymph flow in ears supplied with a pulsating flow of blood is greater than in those perfused at constant pressure, even when both the blood pressure and rate of flow are less. During the present work, when comparing the rate of interstitial dye spread in 17 ears perfused at constant pressure with that occurring in 18 ears supplied with a pulsating flow, the advantage of pressure has been given to the former. For example 9 of the constant pressure perfusions were done at a pressure of 141 mm. of mercury. The pressure in 4 experiments stood at 131 mm., in 3 at 120 mm., and in 1 at 152 mm. of mercury. 6 of the perfusions with pulsatile flow were done at pressures of 141/60 mm. of mercury, 5 at 131/60 mm., 2 at 120/60 mm., 1 at 115/60 mm., 2 at 100/60 mm., and 2 at 95/60 mm. Some of these pressures are higher than those existing in the living animal (8–10), others equal to the normal or lower. Wishing to employ throughout the experiments a diastolic pressure approximately like that in normal rabbits, we selected arbitrarily the pressure of 60 mm. of mercury. In order to obtain sufficient blood flow through the ear to give a normal appearance to the organ under the microscope it was often necessary to employ a "systolic" pressure of 141 mm. of Hg, yet some experiments were done with "systolic" pressures of only 95 or 100 mm. of mercury. As the data below show, the findings

were similar in all the experiments, so the results are not to be attributed to an abnormally high pulse pressure in the experiments involving "systolic" pressures of 141 mm. of mercury. It should be noted that the mean pressure in all the pulsatile perfusion experiments was always far lower than the lowest constant pressure employed. However, in describing the effects of pressure differences upon the findings it has been convenient to employ the figure for the "systolic" pressure of a pulsating flow of blood as if it represented the true pressure. In comparing the results of experiments, for example, in which the "systolic" pulsatile pressure was 120 mm. of mercury and the constant pressure stood at the same figure, the pressures will be called "equal," although as a matter of fact the mean pressure was lower in the instance first mentioned. In cases in which the pulsatile pressure is termed higher than the constant pressure we actually mean that the "systolic" pressure of the former is higher than the latter.

The rate of flow of the perfused blood could be regulated only within small limits, since it varied with the state of dilatation or contraction of the arterial tree of the ear. For example, if the arteries and arterioles of the ear remained in a state of relative contraction the blood flow, whether impelled by pulsatile or constant pressures, remained small. Increased flow could only be attained by an increase in the pressure of the perfusate. But we had determined to keep the pulsatile "systolic" pressures no higher than 141 mm. of mercury. As result, in some experiments the flow of blood was much smaller than in others. In many experiments on the other hand the flow from the perfusion apparatus was too great. This could be reduced at will, as described in the preceding paper (1) by adjusting a screw clamp near the outlet of the perfusion apparatus. The rate of blood flow and the pressures were equal in some experiments, while in others the rates of flow or the pressures of the perfusates, or both, differed greatly. As result, we were able to compare the spread of dye under these varying conditions.

In every case when the final observations had been made, 0.03 cc. to 0.1 cc. of a 21.6 per cent aqueous pontamine sky blue solution, isotonic with blood, was injected through the delivery tube of the perfusion apparatus, to circulate in the vessels of the ear, while the rate of blood flow was measured. The distribution of dye in and about the maculae and the rate at which the color was carried upon the blood to the tissues was noted. Finally the ear, which had been weighed prior to the perfusion, was weighed again, and pieces of tissue removed for section.

During each experiment the appearance of subclinical or of frank edema was carefully sought for. Using a sharp dissecting needle and watching with the binocular microscope, we endeavored to elicit evidence of microscopic pitting upon pressure. In many of the experiments edema occurred and could be recognized in this way. Some ears became intensely edematous, while others showed but the faintest traces of the condition or developed sharply localized edematous patches involving one or more of the dye maculae. All these instances will be considered separately, below.

The Spread of Dye in the Intact Ears of Normal Rabbits

In 8 experiments dye maculae were placed in the ears of normal unanesthetized rabbits sitting quietly in a box, tilted in such a way that the upper surface of the animal's ear containing the dye spots remained horizontal. In each experiment dye was instilled into 6 or 8 regions of the middle and outer thirds of the ear, and the interstitial spread measured as described. Only a few experiments were done, just enough to obtain findings which might serve as a measure of normality for the appraisal of the results obtained in perfusion experiments.

In some of the experiments not included in the 8 mentioned above, the ear under observation became intensely hyperemic. These experiments were ruled out for it was found, in work to be reported later, that in the ear of the mouse the interstitial spread of dye is enhanced by active hyperemia. In other experiments dye appeared in lymphatics or in blood vessels during the periods of observation. These instances too were ruled out, for dye drained away in the vessels instead of spreading interstitially. There remained in the 8 experiments 21 dye maculae that had been placed in ears in which the circulation seemed normal during the experimental period. As already mentioned these dye spots could not be made uniform in size. It was to be expected that large spots would not increase in size as rapidly as small ones. To demonstrate the rate of interstitial dye spread in the normal ear and to rule out the influence of the variations in size of the maculae, we have divided them into groups according to their initial size. The spread of the spots in each group is indicated in Table I, the first group containing maculae less than 50 planimetric units in initial area, that is to say spots originally 0.8 to 1.0 mm. in diameter, the second group containing spots 50 to 100 planimetric units in original area, 1.1 to 1.5 mm. in diameter. The next group included the maculae of 100 to 200 and the last those of 200 to 300 planimetric units, spots varying roughly from 1.7 mm. to 2.4 mm. and from 2.5 to 3.3 mm. in diameter. The data for each group are arranged in three vertical columns, the first showing the initial area of each spot, the smallest at the top, the largest at the bottom, the second and third columns showing the spread of the spots after half an hour and after one hour respectively. As already described the spread is expressed as the number of times the spot increased its initial area. For example in the first group a spot 23 planimetric units in area became 115.5 planimetric units in area after half an hour and 174.4 units after an hour, thereby increasing its initial area 5.0 and 7.6 times. The averages of the initial areas and of the increases in size of each group are given at the bottom of each column.

Inspection of the averages given in Table I shows that, as one would expect, the smaller dye spots increased in size more rapidly than the larger ones. Spots of approximately equal initial size spread more

uniformly. Hence to compare the dye spread in ears perfused with pulsating blood with that taking place in ears perfused with a constant flow we have used only the data from groups of spots having approximately the same initial size.

TABLE I

The Spread of Dye Spots of Differing Sizes in the Ears of Normal Rabbits

Initial area less than 50 planimetric units		Initial area between 50 and 100 planimetric units		Initial area between 100 and 200 planimetric units		Initial area between 200 and 300 planimetric units	
Initial areas	Interstitial spread of dye	Initial areas	Interstitial spread of dye	Initial areas	Interstitial spread of dye	Initial areas	Interstitial spread of dye
Planimetric units	Ratio* of spread	Planimetric units	Ratio of spread	Planimetric units	Ratio of spread	Planimetric units	Ratio of spread
	After ½ hr.	After 1 hr.		After ½ hr.	After 1 hr.		After ½ hr.	After 1 hr.		After ½ hr.	After 1 hr.
23	5.0	7.6	52	3.9	6.6	101	4.7	8.5	287	2.7	3.4
29	5.2	9.0	59	4.0	8.1	107	3.1	4.9			
39	3.4	6.8	63	3.5	6.4	115	2.8	4.9			
42	4.6	6.1	77	5.9	5.6	120	3.3	4.3			
43	5.2	9.6	78	3.1	5.8	142	2.6	4.6			
			96	4.8	6.5	159	5.2	7.9			
						163	3.5	5.9			
						182	3.6	5.1			
						198	2.5	3.4			
Average..35	4.7	7.8	78	4.2	6.5	143	3.5	5.5			

* See text and legend.

The table compares the interstitial spread of dye spots of differing sizes in the ears of normal rabbits. The spots have been classified in 4 groups according to their size as measured in planimetric units (see text). The data from each group have been arranged in three vertical columns, the first showing the initial area of each dye spot, the smallest at the top, the largest at the bottom. The second and third columns in each group represent the spread of the spots after ½ hour and 1 hour respectively. The spread has been presented as the ratio of the area of a given dye spot after 30, or 60, minutes to its initial area. For example, when it was found that the area of the first spot in group 1, after 30 minutes, was 5 times larger than at the beginning of the experiment, the spread was expressed as 5.0.

It will be noted that the smaller spots spread more rapidly than the larger ones.

The Spread of Dye in Amputated and Perfused Rabbit Ears

The Spread of Dyes through Edema-Free Tissues.—In 49 experiments the ears of rabbits of about 2 kilos were amputated and perfused with

defibrinated rabbit's blood, as described in the preceding paper. During the perfusions, 6 or 8 dye maculae were placed in each ear, in all 274 of them, 129 in 24 ears perfused with pulsatile flow and 145 in 25 ears perfused at constant pressures. Of the 49 experiments 35 were free from objection on the scores already mentioned and in these the rate of enlargement of 184 dye spots was measured for periods of an hour, 72 spots in 18 ears perfused with pulsating blood and 112 in 17 ears perfused at constant pressure.

The spread of these spots has been considered after separating the data into comparable groups in relation to the initial size of the dye spots, the pressure and the rate of blood flow per gram of ear, and the occurrence of local or general edema. In about 30 per cent of the experiments local or general edema appeared in the perfused ears and was demonstrated under the binocular microscope by pressure with a dissecting needle. The findings in these experiments will be described separately below.

The data showing the spread of the dye spots in edema-free tissue have been graphically depicted in Chart 1 by plotting the average spread of groups of similar sized dye spots along the ordinate and their initial measurements along the abscissa. As already done in the experiments on normal animals, the spots in both pulsatile and in constant pressure experiments were divided into groups according to their original size. The first group included spots up to 50 planimetric units in initial area (0.8 to 1.0 mm. in diameter), the second group those between 50 and 100 (1.1 to 1.5 mm. in diameter), the next 3 groups, those of 100 to 200, 200 to 300, and 300 to 400 planimetric units. In the experiments employing pulsatile perfusion, the spots of dye initially less than 50 planimetric units in area averaged 24 units in area. After spreading for one hour, the areas had increased on the average 8.2 times. To represent this fact in the chart, a point (marked with a cross, x) was located 24 units along the scale of the abscissa and 8.2 units along the scale of the ordinate. Spots of dye originally more than 50 and less than 100 planimetric units, averaging 74 units, increased in area on the average 5.9 times. The curve therefore runs through the next point, 74 on the abscissa, 5.9 on the ordinate. In this way each group's average of initial size and final spread has been plotted. Similarly the averages of the initial areas and the spread of the groups of spots in ears perfused at constant pressure have been plotted in small black circles (●). To compare the spread of the dye spots in ears perfused with pulsatile and with constant currents, we have charted the heavy, continuous line, marked P, representing the findings in the ears perfused with pulsatile current, and the light, continuous curved line, C, representing those obtained in the constant pressure experiments. Both lines show that in both types of perfusion the smaller

maculae spread relatively faster than larger ones. They show further the fact that, in each group of similar initial size, spread was always greater in the ears perfused with pulsating blood. For comparison we have also plotted in the
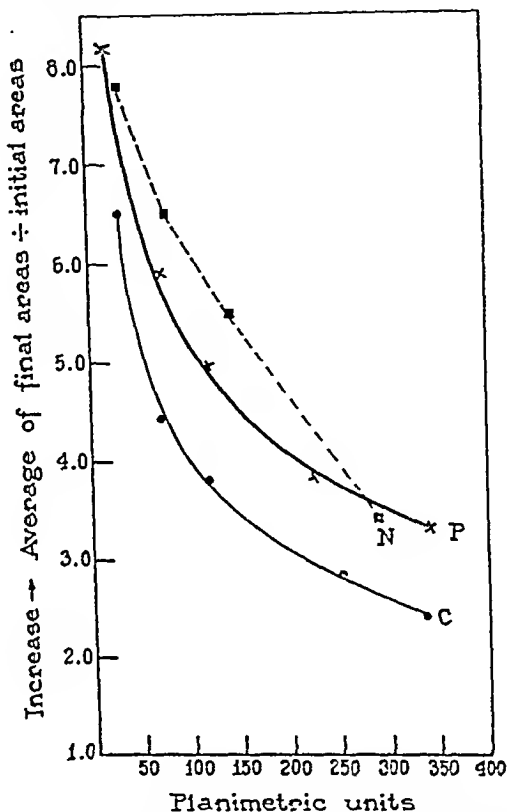
CHART 1. *The Interstitial Spread of Dye Spots of Differing Sizes in the Ears of Normal Rabbits and in Amputated Ears Supplied either with a Pulsatile Flow of Blood, or with a Constant Flow.*—The heavy continuous line, P, represents the spread of dye maculae in ears perfused with a pulsating flow of blood. The fine continuous line, C, shows the spread of maculae in ears perfused with blood at constant pressure. The dotted line, N, shows the spread of dye spots in the ears of normal rabbits. The manner in which the curves are derived is fully described in the text.

same manner on Chart 1 the data from Table I, showing by small squares (■) the averages of the initial areas and the spread of each of the four groups of dye spots in that table. The resulting dotted line, N, connecting these squares represents the spread of the groups of similar sized dye maculae in the ears of normal

rabbits.   Spread of dye in the normal ear was slightly greater than in the amputated ears perfused with pulsatile blood flow but the difference was not marked.

A table like Table I was constructed to show the spread of individual spots of the various groups of maculae in the amputated ears.   This has not been included in the paper, but it should be noted that the spread of dye spots in the perfusion experiments was far more regular than in the normal ears.   This difference is due, no doubt, to
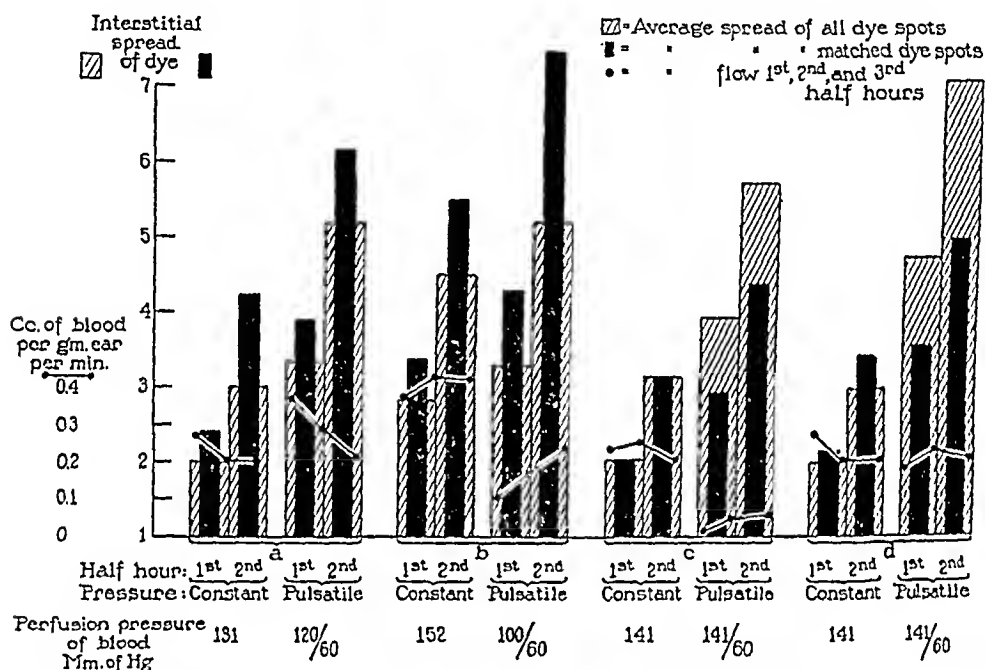
CHART 2. *The Effect of the Pulse on the Interstitial Spread of Dye.*—The chart is fully described in the text.

the fact that the conditions of blood flow and pressure in the normal ears varied much more than did the conditions of flow and pressure in the perfused ears.

Chart 1 shows that the relative rate of spread of the dye spots varied inversely as their size in both types of experiment and that, on the average, maculae of approximately the same size spread more under the influence of pulsation than in its absence.   From the chart it is clear, too, that were one to make maculae of dye all under 50 planimetric units (0.8 to 1.0 mm. in diameter), and to compare their

spread exclusively with large ones, for example 300 to 400 planimetric units in area, over 3.3 mm. in diameter, the influence of size would affect their relative rate of increase to such an extent that the physiological difference under study would not be apparent. Actually in performing the experiments one never made all large or all small maculae: inevitably the spots varied much in size. From the findings to be described below it will be obvious too that a fairly even distribution of maculae of the different sizes occurred in both kinds of experiment. The average spread of all maculae of various sizes in all the ears perfused with pulsating blood, with rapid or slow flow, was 6.1 times the average of their initial areas. Maculae in ears perfused at constant pressures spread on the average 4.1 times. Yet the average rate of blood flow in the constant pressure perfusions was 150 per cent greater than in the pulsatile perfusions.

To bring out the important influence of a pulsatile current we have plotted in Chart 2 the data from 8 typical experiments grouped as 4 sets, *a*, *b*, *c*, and *d*, each of which represents the measurements taken in one ear perfused with a constant flow of blood and in another served with a pulsating current of blood. These 8 experiments were selected for comparison, and paired as shown for the reason that in each pair of ears there could be found at least 3, or more, maculae of approximately the same size, while at the same time, in the same ears, other maculae were present which varied greatly in size.

In Chart 2 each set of four broad cross-hatched columns with narrow solid black columns included within them represents the data from one of the comparisons as described above. The data from the constant pressure perfusion stand in the first two columns at the left in each set of four, those from the pulsatile pressure perfusion, to be compared with it, in the two columns at the right. The first and second of the broad, cross-hatched columns in each set of four show the average spread for the first and second half hours respectively of all dye maculae, usually 6 or 8 of various sizes placed in the ear perfused with constant flow. The third and fourth broad columns in each set represent the same findings in the ear perfused with pulsating blood. All the broad, cross-hatched columns represent the spread of dye spots unmatched in size. We have contrasted with this, by means of the narrow black columns, the spread of dye spots of approximately the same initial size in the same ears perfused at constant and pulsatile pressures, selecting for comparison, experiments in which we were able to pick out from each ear at least 3, and sometimes even 6 pairs of spots, all of which were practically the same in initial size. As just mentioned the narrow columns

represent the average spread of these matched pairs of spots. The first narrow solid black column represents their spread in the first half hour, the second, for the next similar period. The rate and the variations in the flow of blood per gram of ear tissue have been shown by the heavy continuous lines joining black dots. The latter represent the upper and lower limits of the variations in flow during each half hour period during which the spread of dye was measured. The lines therefore indicate roughly the rate of flow during each period. The pressures employed for each constant pressure experiment, as indicated, were always as high or higher than the "systolic" pressure in the pulsatile perfusions.

TABLE II

The Spread of Dye Spots of Approximately Equal Size in the Perfused Ears of Rabbits

Perfused at constant pressure			Perfused at pulsating pressure			Non-perfused ears of dead animals		
Initial area	Interstitial spread of dye		Initial area	Interstitial spread of dye		Initial area	Interstitial spread of dye	
Planimetric units	Ratio* of spread		Planimetric units	Ratio of spread		Plani-metric units	Ratio of spread	
	After ½ hr.	After 1 hr.		After ½ hr.	After 1 hr.		After ½ hr.	After 1 hr.
109	2.2	3.2	111	2.8	4.5	100	2.0	2.6
110	2.6	3.3	112	2.7	4.9	111	1.9	2.5
111	2.5	3.5	118	3.6	5.0	112	1.9	2.6
112	3.3	5.0	119	4.3	8.7	117	1.9	2.7
112	2.6	3.9	119	2.4	4.8	120	1.9	2.6
114	2.2	3.6	121	2.8	4.2	122	1.8	2.5
120	2.9	3.8	123	2.6	3.6	123	2.1	2.5
124	2.4	3.0	124	3.0	4.5	124	1.9	2.6
Average..114	2.6	3.7	118	3.0	5.0	116	1.9	2.6

* The table is fully described in the text.

In every comparison the unmatched dye spots spread farther when pulsatile currents were used. The spread of the matched spots of equal size showed still greater differences. As the experiments were selected in order to compare the spread of dye spots of equal size, the differences in rate of blood flow and pressure during the perfusions varied greatly. Some consistent findings resulted, for example in Chart 2 *d*, the blood flow in each ear was about equal all through both experiments and the pressures were 141 mm. and 141/60 mm. of mercury. At equal pressures and equal rates of flow the spread of

the unmatched dye spots (cross-hatched columns) and also that of the spots matched in size (narrow black columns) was greater in the ear perfused at pulsatory pressure. In the pairs of experiments shown in b and c the flow during the pulsatile pressure perfusions was small compared with that of the constant pressure perfusions, as shown by the lines crossing the columns. In c the pressures were "equal," being 141 and 141/60 mm. of mercury, in b the pulsatory pressure was lower than the constant pressure. Nevertheless the spread of both matched and unmatched dye spots was greater in the pulsatile perfusion experiments. In the pair of experiments compared in Chart 2 a, the blood flow during the pulsatile perfusion was greater than that during the constant pressure perfusion but the pulsatory pressure of the former, 120/60 mm. of mercury, was lower than that of the constant pressure experiment, 131 mm. of mercury. Dye spread was greater in the pulsatile perfusion experiment.

It is clear from these findings that the effect of pulsation upon the spread of dye solution was sufficiently great to overcome all the other variable influences, such as differences in size of the dye spots, rate of perfusion flow or its pressure.

To show the effect of pulsation upon the interstitial spread of dye, we have compared in the first two sections of Table II the spread of 16 dye spots, 8 from ears perfused with pulsating blood and 8 from ears perfused with constant flow. All had initial areas of more than 100 and less than 125 planimetric units, that is to say the spots were almost the same size, varying only from 1.7 to 2.0 mm. in diameter. The particular limits, 100 to 125 planimetric units, were selected because the differences in size were small enough to yield maculae of comparable area, and more maculae happened to fall within these limits than in those of any other similarly narrow range. All the spots had been placed in ears that remained free from edema, and the experiments were free from technical errors. A comparison of the spread of these dye spots has been made in the table without regard for the differences in the rates of flow of the perfused blood or its pressure. For comparison the table shows also the spread of 8 maculae of similar size introduced into the non-amputated ears of rabbits 2 to 3 minutes after they had been killed with ether. In the latter the spread of dye was about two-thirds as great, at the end of one

hour, as in the ears perfused at constant pressure, and about half as great as in the ears perfused at pulsating pressure.

In a few instances we were able to compare the spread of dye maculae of equal initial area in ears which happened to be perfused with equal volumes of blood per gram of tissue, and at "equal" constant

TABLE III

A Comparison of the Spread of Dye Maculae of Nearly the Same Size in Ears Perfused with a Constant or a Pulsating Flow of Blood at Nearly Equal Rates of Flow and at Equal Pressures*

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(1a)	(2a)	(3a)	(4a)	(5a)	(6a)	(7a)
	Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread			Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread	
Exp. No.	Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.	Exp. No.	Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.
I	23	141	0.29	0.28	3.8	5.1	1	25	141/60	0.25	0.24	8.4	13.0
II	48	120	0.19	0.23	1.7	3.2	2	52	120/60	0.20	0.21	4.0	6.3
III	64	131	0.28	0.30	2.2	3.7	3	64	131/60	0.23	0.28	4.1	6.0
							4	66	131/60	0.25	0.22	4.2	5.8
IV	70	141	0.26	0.21	2.4	4.2	5	74	141/60	0.22	0.28	3.8	5.2
V	87	141	0.24	0.23	1.9	3.7	6	93	141/60	0.27	0.30	3.3	5.4
VI	96	120	0.18	0.20	2.0	3.6	7	99	120/60	0.16	0.19	3.7	5.9
VII	116	141	0.28	0.30	2.9	3.9	8	116	141/60	0.35	0.24	2.4	4.8
							9	124	141/60	0.28	0.33	3.0	4.5

The table, fully described in the text, shows that dye spread more rapidly in tissues perfused with a pulsatile flow of blood than in those perfused at constant pressure.

* "Systolic" pressure of the pulsatile perfusion equal to constant pressure.

and pulsatile pressures, that is to say, at a constant pressure equal to the "systolic" pressure of the pulsating perfusate (Table III). In some instances the spots compared were of slightly unequal size, and in such instances we selected from the constant pressure perfusions only those spots which were smaller than those from the pulsatile

perfusions with which they were matched. The former should have spread relatively faster than the latter, other things being equal. Only a few comparisons were possible under these rigid conditions. The spots used for Chart 2 *d* have not been included in the table.

Columns 1 to 7, placed to the left of the double lines in the center of Table III, show the data obtained from 7 spots of dye in ears perfused at constant pressure. In the vertical column 1 we have given distinguishing Roman numbers to each dye spot. The vertical columns 2 to 7 show, respectively, the initial areas of these maculae in planimetric units, the pressure of the perfusate, the rate of blood flow per gram of ear in the half hour periods of the experiment, and the spread of the dye spots during these periods. The data concerning these spots have been compared in the right half of the table, columns 1*a* to 7*a*, with similar data on the spread of 9 dye spots found in ears perfused with pulsating blood, the "systolic" pressure of which equalled the pressure employed in the perfusions at constant pressure, while the rate of blood flow differed but little. Column 1 *a* contains distinguishing numbers for these spots. Between each set of horizontal spaces appear the comparable data, that is to say, the spread of each spot in an ear perfused at constant pressure is compared with that of one spot, or occasionally two spots, of almost equal size in ears perfused at pulsatile pressure. For example, comparing the spread of dye spot III with that of spots 3 and 4, the constant pressure, column 3, equalled the "systolic" pressure in the experiments with pulsating current, from which the 2 spots were taken, column 3 *a*. The flow of blood in the constant pressure perfusion, columns 4 and 5, was slightly greater than the flow in both of the pulsatile pressure perfusions, columns 4*a* and 5*a*. Dye spot 3 in an ear perfused with pulsating current, spread 4.1 times its initial area in half an hour, and 6.0 times in an hour, columns 6*a* and 7*a*. Dye spot 4 spread 4.2 and 5.8 times in similar periods. These figures are to be compared with those in columns 6 and 7 respectively, showing the spread of the dye spot in the ear perfused with a constant flow of blood, during equal time intervals. In a similar manner the other comparisons can be made.

The table shows that dye spread more rapidly in the tissues perfused with a pulsatile flow of blood than in tissue perfused at constant pressure, when the rates of flow were equal and the "systolic" pressure of the pulsatile perfusion equalled the constant pressure of the compared experiment.

The Effect of Variations in the Flow of the Perfused Blood.—As already mentioned, the rate of flow of the perfused blood varied much from one experiment to another, at times by accident, at times by design. Nevertheless in the ears perfused with pulsatile flow, dye spread more rapidly than in those perfused with a constant current,

TABLE IV

The Spread of Dye Maculae of Nearly the Same Size in Ears Perfused with a Constant or a Pulsatile Flow of Blood at "Equal" Pressures but at Greatly Differing Rates of Flow

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(1a)	(2a)	(3a)	(4a)	(5a)	(6a)	(7a)
	Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread			Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread	
Exp. No.	Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.	Exp. No.	Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.
I	19	131	0.30	0.26	3.6	5.1	1	25	131/60	0.21	0.22	6.3	9.2
II	64	120	0.26	0.32	2.3	3.8	2	68	120/60	0.22	0.19	4.4	6.4
III	74	141	0.29	0.28	2.1	3.6	3	76	141/60	0.16	0.13	4.0	6.2
							4	79	"	0.12	0.13	4.2	6.3
IV	93	141	0.31	0.32	2.4	4.0	5	94	141/60	0.19	0.20	3.8	5.2
							6	103	"	0.05	0.06	3.8	5.6
V	134	141	0.11	0.13	2.8	3.7	7	133	141/60	0.08	0.11	4.3	6.3
							8	143	"	0.05	0.06	3.8	5.6
VI	73	141	0.21	0.22	2.6	3.6	9	77	141/60	0.41	0.23	3.4	5.4
							10	79	"	0.23	0.28	4.4	6.6
VII	77	120	0.18	0.23	2.1	3.6	11	83	120/60	0.23	0.28	3.7	5.6
							12	84	"	0.27	0.30	3.9	5.8
VIII	77	131	0.11	0.13	2.3	3.5	13	79	131/60	0.31	0.33	4.4	6.4
							14	81	"	0.23	0.28	3.8	6.0
IX	108	141	0.19	0.18	2.5	3.3	15	114	141/60	0.22	0.24	4.1	5.2
							16	116	"	0.35	0.23	2.4	4.8
							17	124	"	0.28	0.33	3.0	4.5
X	109	131	0.18	0.21	2.7	3.8	18	124	131/60	0.25	0.21	3.4	5.8
							19	126	"	0.22	0.23	4.0	6.5
XI	116	141	0.28	0.30	2.1	3.8	16	116	141/60	0.35	0.23	2.4	4.8
							17	124	"	0.28	0.33	3.0	4.5
XII	126	131	0.12	0.13	2.4	3.0	18	124	131/60	0.25	0.21	3.4	5.8
							19	126	"	0.22	0.23	4.0	6.5

The table, as described in the text, shows that dye spread more rapidly in the ears perfused with a pulsating current of blood, in spite of great differences in the rate of blood flow. The pressures were equal.

even when the flow in the former was but one-quarter that in the latter. To illustrate this fact further, we have presented in Table IV data showing the spread, in groups, of all the dye spots of almost the same size that could be found in experiments in which the constant pressures and "systolic" pulsating pressures were equal, but in which the rates of flow differed greatly. The number of maculae that fulfilled these conditions and could be compared was limited; the table contains the data from all of them, 12 from constant pressure experiments and 19 from pulsatile flow perfusions.

The plan of the table is like the preceding one. In each section of the left side of the table and between the horizontal spaces appear the data on the spread of single dye spots in ears perfused at constant pressure. They are to be compared with the data in the right half of the table lying between the same horizontal spaces. These figures give the data concerning the spread of those maculae comparable in initial size, or very slightly larger, which were found in ears perfused at pulsatile pressures (the "systolic" impulse of which equalled the constant pressure) but with differing volumes of blood per gram of tissue per minute. The first section of the table compares the spread of maculae during perfusions in which the pulsatile flow was less than the flow at constant pressure. The second section shows the spread when the pulsatile flow was greater. In the table, the data from certain dye spots reappear from time to time and can be recognized by the distinguishing number given to each spot.

In all these comparisons the spread of dye spots in constant pressure experiments has been compared as usual with that of equally large or slightly larger spots from pulsatile pressure experiments. The advantage is therefore given to the constant pressure experiments as already described. A glance at the figures in columns 6 *a* and 7 *a*, comparing them with the figures in columns 6 and 7, shows that the maculae in the pulsatile perfusion experiments spread more rapidly than those in the constant pressure perfusions regardless of the differences in the rate of blood flow. For example, in the comparison of the spread of dye spots IV and 6, the volume of flow of the pulsating perfusate per gram of ear was but 1/6 that in the constant pressure perfusion with which comparison was made; nevertheless dye spread was much greater in the former.

The Effects of Variations in Pressure.—The findings so far reported show further that the greater dye spread in ears perfused with a pulsatile blood flow cannot have been due to the pressure of the latter.

For example, the comparison in Chart 2 b shows a greater dye spread in an ear perfused with a pulsatile flow than in one perfused with a constant flow although both the pressure and the rate of flow in the former were lower than in the latter. In this connection there is one comparison which can be made from our data which is of additional interest. A perfusion, done at a pulsatory pressure of 95/60 mm. of mercury, resulted in the circulation of only 0.11 cc. and 0.12 cc. of blood per gm. of ear per minute, in the first and second half hour

TABLE V

The Spread of Dye Maculae of Nearly the Same Size in One Ear Perfused with a Constant Flow of Blood and in Another Ear Perfused with a Pulsatile Flow of Blood. Equal Rates of Flow but Different Pressures Were Used

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(1a)	(2a)	(3a)	(4a)	(5a)	(6a)	(7a)
All spots in the same ear	Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread		Both spots in the same ear	Initial area	Pressure of perfusate	Rate of blood flow per gram of ear per minute		Ratio of spread	
	Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.		Planimetric units	mm. Hg	In first ½ hr.	In second ½ hr.	After ½ hr.	After 1 hr.
	74	141	0.14	0.12	2.8	3.7		61	95/60	0.11	0.12	4.3	6.3
	78	141	0.14	0.12	2.8	3.7							
	111	141	0.14	0.12	2.5	3.5		116	95/60	0.11	0.12	4.3	8.7
	110	141	0.14	0.12	2.6	3.3							

This table, described in the text, gives the data from 2 experiments in which it was possible to compare the spread of dye in one ear perfused with a pulsatile current of blood at a low pressure, with the spread of dye in another ear perfused at a higher constant pressure but with an equal volume of blood per gram of tissue per minute. The spread of dye was greater in the ear supplied with a pulsatile flow of blood.

periods, respectively. We are able to find one experiment done with constant pressure in which the perfusion rate was comparable, but in which a higher perfusion pressure, 141 mm. of mercury, had been used. Fortunately, 4 of the dye spots in the latter compared closely in size with 2 in the former. The data concerning their spread are shown in Table V. Despite the lower pulsatile pressure, dye spread was greater in the ear perfused in this way. It is to be noted too that in this experiment the pulse pressure was small.

The Effect of Edema

So far the findings in only two-thirds of our experiments have been considered, for in the other third edema appeared in the tissues of the perfused ears despite all efforts to avoid it. Edema occurred more often in the experiments involving constant pressure perfusion than in those done with pulsations. It appeared frequently in the perfusions done at the highest pressures and with the greatest flow of blood, but this finding was by no means regular. No single group of circumstances could be found which seemed to favor its appearance. We took the opportunity to study the spread of dye during the formation of edema in the amputated ears while they were perfused with a constant or pulsatile flow of blood. The studies were made as already described save that the maculae of dye were placed in regions of the ears already edematous and becoming more so, or in skin that happened to become edematous a few minutes later. The findings need no detailed report. In 13 perfused ears in which edema appeared, more than 95 maculae had been made. The spread of only 77 of these was measured, 36 from pulsatile perfusions and 41 from those done at constant pressures. The remainder were ruled out because the dye solution together with edema fluid oozed out of the tissue on the surface of the skin.

In Chart 3 we have plotted the spread of these maculae in the same manner as in Chart 1, dividing the spots into 5 groups according to their initial sizes.

The heavy continuous line, PE, represents the spread of dye in the ears which became edematous during the pulsatile perfusions, the heavy dotted line, CE, the dye spread during the perfusions at constant pressure. We have reproduced in the same chart the curves from Chart 1 representing the dye spread in nonedematous ears, the light continuous line, P, showing that which occurred during the pulsatile perfusions, the light dotted line, C, that taking place during perfusions at constant pressure.

As the chart shows, dye spread most in the ears which became edematous during perfusion with a pulsatile flow of blood, line PE. Spread was slightly greater than in ears similarly perfused but not edematous, line P. By contrast the positions of lines CE and C, in the chart, demonstrate an important fact; the onset of edema adds but little to the rate of dye spread in ears perfused with constant

pressure.   Indeed the spread of dye in these edematous ears was less than that occurring in non-edematous ears perfused with a pulsatile flow of blood.   More will be said of this below.   It is to be stressed that our work has allowed us to study the dye spread only during the formation of edema, not after its formation.   In work to be
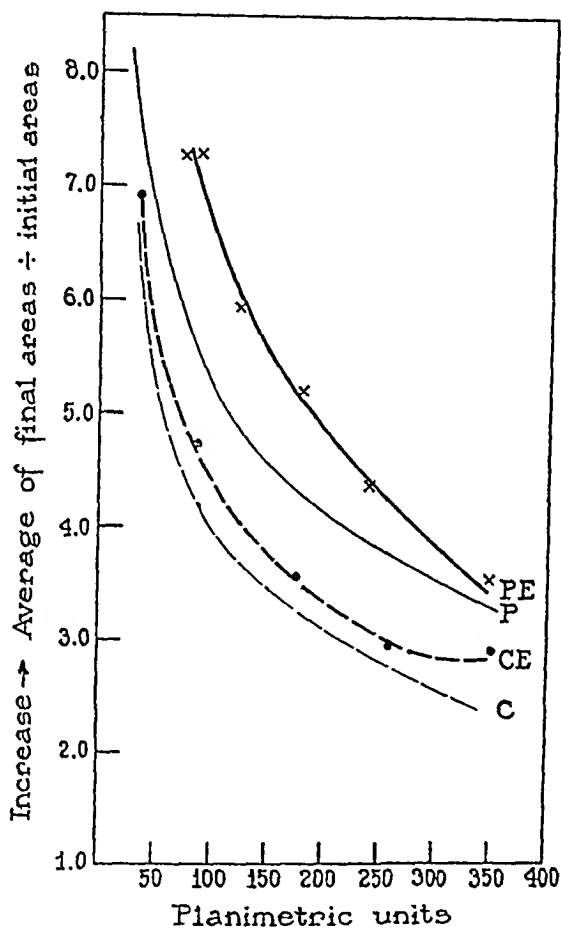
CHART 3. *The Spread of Dye Spots in Ears Becoming Edematous While Perfused with a Pulsatile or a Constant Flow of Blood.*—The chart is described in the text.

published it has been shown that dyes introduced into the connective tissue in the same way as in the present experiments spread farther in tissues becoming edematous than in those already boggy.   Similar findings would doubtless have appeared had a comparison been made in the course of the present work.

The Effect of Pulsation of the Vessels upon the Removal of Dye from the Tissues

It was frequently noticed, toward the end of the hour during which the measurements were made, that the dye maculae lost some of their color, not only near their margins but throughout. This occurred chiefly in the tissues perfused at pulsatile pressure. In these instances, too, it was usually noted that the apparent spread of the dye through the tissues was far less in the second half hour than in the first, and when, upon a few occasions, measurements were continued for a third half hour period, the spot of color often became smaller as well as paler. Obviously the dye was being rapidly removed from the tissues. These phenomena appeared usually in the pulsatile perfusion experiments and seldom, and then only dubiously, in the constant pressure perfusions. It seemed wise, therefore, to test the effect of the pulse upon the rate of absorption of dye from the tissues. To do this a total of 48 maculae were made in the usual manner in 14 of the experiments described above, employing a highly diffusible dye, patent blue V, which we have used in many previous studies of the physiology of lymphatics of animals (5–7) and of man (2–4). The spread of these maculae was observed and measured as already described. A constant pressure of 141 mm. of mercury and a pulsatile pressure of 141/60 mm. of mercury were selected for the experiments. In all those from which data are considered here the blood flow was greater when constant pressures were used. The diffusible dye spread with great rapidity so that the spots often became too pale to measure accurately after an hour. Furthermore, edema appeared in the ears in about one-third of these experiments. As result, in only 24 instances could the spread of these maculae be measured accurately for periods up to one hour in ears which did not become edematous. 13 of these dye spots were made in ears perfused at constant pressure and only 11 in the pulsatile perfusion experiments. Nevertheless these scanty data showed such a constant difference that further experimentation was considered unnecessary.

In Table VI the data have been arranged as in Table I to show the spread of the dye spots after 30 minutes and after 1 hour. When pulsatile perfusion was done the average of the areas of the 11 maculae, varying in original size from 77 to 180

planimetric units, increased 5.9 times within 30 minutes. In the next similar period, however, the average of their areas decreased, becoming only 4.3 times the average of the initial areas. Of the 13 maculae, in ears perfused at constant pressure, the initial areas of 8 fell within the limits of the initial areas of the maculae in the ears perfused with pulsation. The data gathered from these instances are enclosed in the box of heavy lines in the table. The average of the areas of these 8 maculae was found to be 4.4 times that of the average of their

TABLE VI

The Spread of Maculae of a Highly Diffusible Dye, Patent Blue V, in Ears Perfused with a Pulsatile or a Constant Flow of Blood

Perfused at pulsatile pressure			Perfused at constant pressure		
Initial area	Interstitial spread of dye		Initial area	Interstitial spread of dye	
	Ratio of spread			Ratio of spread	
Planimetric units	After ½ hr.	After 1 hr.	Planimetric units	After ½ hr.	After 1 hr.
77	7.4	7.4	68	5.1	6.6
84	5.0	4.9	82	4.7	6.9
87	6.0	3.9	85	5.0	7.5
97	5.3	2.4	94	5.2	7.3
113	6.5	4.0	99	4.7 Average, 4.4	7.0 Average, 6.4
121	5.6	4.1	113	5.2	5.0
135	6.1	4.8	132	3.0	5.4
151	7.1	5.0	166	5.0	6.2
155	5.8	2.9	178	6.1	5 8
173	4.5	3.9	188	5.2	7.0
180	6.2	4.0	213	3.3	4.5
			275	4.4	5.0
			370	2.6	2.8
Average.....	5.9	4.3		4.6	5.9

The table is fully described in the text.

initial areas after spreading for ½ hour. During the second half hour of the experiments the average increased to 6.4 times the initial average figure.

An average of the spread of all the maculae in these constant pressure experiments, regardless of whether the initial areas were greater or smaller than those of the maculae in the pulsatile perfusion experiments, also shows, during the second half of the experiments, a continued increase in size from 4.6 to 5.9 times the average of their initial areas. The dye spots in the constant pressure experiments did not decrease in size as did the maculae of the pulsation perfusions.

These measurements do not adequately portray the fact which was clearly recognizable to the observer, that dye absorption was far more rapid in the ears perfused with a pulsating flow of blood. As already mentioned many of the maculae watched under such conditions became too pale to measure, and all were far paler at the end of the experiments than the dye spots in the ears perfused at constant pressure. Clearly the pulsation of the blood vessels enhanced dye removal from the tissues as well as spread.

DISCUSSION

It is recognized that the movement of dyes or of dye-colored solutions through a tissue may not be representative of the movement of other substances or fluids in the same tissue. Nevertheless, in the absence of better means, it has seemed important to determine the effect of the pulse upon the interstitial spread of certain dyes. The findings reported here have shown that the pulsation of blood vessels in the ear of the rabbit increases the spread of dye through the skin of the organ after its introduction into the connective tissue.

What can be said of the effect of the pulse upon the interstitial movement of those substances and fluids which are concerned with tissue nutrition? The preceding paper (1) has shown that the pulsation of blood vessels increases the formation and the flow of lymph, that is to say increases the movement of fluid through the tissues. Pulsation also increases the interstitial spread of dyes. One might assume that the pulsation of blood brings about a greater escape of fluid or material from the blood to the tissues, each impact of the pulsation pushing blood constituents against those already passing from the vessel and present in its wall. In this way, particles, large molecules, or fluids might be forced into the tissues like grain thrown repeatedly against a sieve which would let but little by if the grain were merely pressed against it. Materials already present outside the vessel wall, receiving the impact, would be forced to move through the tissues. It should be noted, however, that the effect of the pulse is negligible or absent in those vessels in which the permeability is greatest, namely, the capillaries, and pulsations are strong only in the relatively thick-walled arterioles and arteries. Some other explanation of our results seems to be called for.

Several findings in this and in the preceding work bear directly upon this problem. Experiments involving pulsatile perfusion with but 1/6 or 1/5 the flow and far lower pressures than those used in constant pressure perfusions invariably yielded both greater lymph flow and greater interstitial dye spread. Can one suppose that fluid escape from the blood vessels was greater under the former than under the latter condition? Further, the effect of pulsation in spreading dye and increasing lymph flow is not due wholly to an increase in the fluid content of the tissues. As already stated, in non-edematous ears the formation and flow of lymph was much increased by pulsation (1). When edema was present in the tissues lymph flow and formation were not greatly increased unless there was pulsation. When edema was present in a tissue perfused with a pulsatile flow lymph formation was greatest. It would seem from this that the movement of fluid through the tissues to form lymph required the mechanical effect of the pulse, the mere presence of excess interstitial fluid failing to bring about this result. In this relation it should be mentioned that, in the present work, edema appeared more frequently in ears perfused at constant pressure than in those supplied by pulsating blood; under the former circumstances the gain in weight was greater than under the latter. Whether more fluid escaped from the vessels in the constant pressure perfusions we cannot definitely say, though it seems unlikely. Certain it is that the return of fluid from the tissues to the blood or lymph was aided by pulsation. The more rapid absorption of dye from the tissues supplied by a pulsatile blood flow corroborated this finding.

From the data of the present paper we can infer that, just as the mechanical influence of the pulse is more important than the changes in the fluid content of the tissues in producing lymph flow, so is it more important, as just discussed in the preceding paragraph, in producing interstitial dye spread. The same conditions which increased one increased the other. For example, in non-edematous ears dye spread was much increased by pulsation; when edema was already present dye spread was not greatly increased unless there was pulsation; when excess fluid was present in a tissue perfused with a pulsatile flow, dye spread was greatest.

How can these findings be explained?   Other work from this laboratory now in press, demonstrates the importance of mechanical factors in the interstitial movement of dye in the ear of the living mouse. It becomes far more rapid when the tissue is subjected to gentle intermittent changes in external pressure, changes far smaller than those occurring in the blood vessels and equivalent to those produced by columns of water only 2 to 8 cm. in height.   These slight changes in pressure applied to the skin of the ear and not from within the vessels as in the present work, spread dye through the tissues with great rapidity.   From this it is clear that to explain our findings one need not invoke the action of the pulse within a vessel forcing materials through its walls.   If slight changes in pressure applied externally to the tissues spread dye through them rapidly it seems possible that the change in caliber of vessels within a tissue can produce a similar effect.   A discussion of the probable mechanism by which the pulse increases the formation and flow of lymph and the spread of dye in the tissues must be postponed for following papers in which further data will be presented concerning the spread of substances through tissues.   Suffice it to state that our experiments show that pulsation of the blood vessels in the perfused rabbit's ear leads to greater formation and flow of lymph, to greater interstitial spread of dye, and to the more rapid absorption of dye from the tissues.

SUMMARY

The pulsation of blood vessels in the ear of the rabbit greatly increases the rate of the spread of dye introduced into the subcutaneous tissue.

The appearance of edema in tissues perfused at a constant pressure leads to very little increase in the rate of dye spread.   By contrast, a rapid interstitial spread of dye occurs in tissues becoming edematous while perfused with a pulsatile flow of blood.

The significance of these facts is discussed.

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THE BEHAVIOR OF POX VIRUSES IN THE RESPIRATORY TRACT

I. The Response of Mice to the Nasal Instillation of Vaccinia Virus

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PLATE 12

Our earlier studies on fowl coryza and mouse catarrh (1, 2) had dealt with infective agents which were essentially specific for the mucous membrane of the respiratory tract. It seemed of interest to extend this work to diseases in which the causal agent provoked an initial coryza but was not necessarily restricted to the respiratory tract. The pox viruses were chosen as being illustrative of this situation and also because they afforded an opportunity for an intimate comparison of the elementary bodies and the coccobacilliform bodies. Aside from observations on the rabbit, little attention appears to have been paid to the upper respiratory tract, in experimental studies of the pox viruses, in spite of its obvious importance as a portal of entry under natural conditions.

The production of a coryza in rabbits by vaccinia virus is well established. Gordon (3) found that the nasal instillation of vaccinia virus in doses containing as little as 0.01 mg. of calf lymph gave rise to a nasal catarrh after 6 to 8 days. There were no general symptoms. Rabbits tested 10 days after recovery were protected against vaccinia virus administered cutaneously. Nicolau and Kopciowska (4) noted that a coryza was a characteristic feature of a spontaneous epizootic attributed to vaccinia. Rosahn. Hu, and Pearce (5) observed that a coryza with a nasal discharge was a prominent manifestation in rabbits experimentally infected with a vaccinia-like virus from a natural outbreak.

The literature on vaccinial pneumonia in rabbits has been reviewed by Armstrong and Lillie (6). They described a highly fatal and characteristic pneumonia

in the rabbit produced by a selected strain of vaccinia virus following intranasal or intratracheal injection. The virus was carried through 8 successive lung transfers with no apparent loss in infectivity.

In the present paper observations are presented on the susceptibility of the white mouse to vaccinia virus introduced by the nasal route.

The initial strain of virus used in this work was a suspension of elementary bodies originally obtained through the courtesy of Dr. Thomas Rivers. The virus was propagated in the chorio-allantoic membrane of embryonated 10 day hen eggs incubated at 37°C. for 2 to 3 days. The method of inoculation was essentially that of Burnet (7). Membranes which showed a characteristic vaccinial reaction and numerous elementary bodies were removed, finely ground, and each suspended in 2 to 5 cc. of saline solution. These suspensions were used both for egg passages and mouse injection. The history of the mice employed has been given elsewhere (2). The mice were usually infected in groups of 5 which were kept together in one cage and held in a quarantine unit. The virus was introduced into etherized mice either by dropping a suspension on the nose or by dipping the nose directly into the suspension.

The introduction of vaccinia virus into the nasal passages of mice was regularly followed by a coryza, and later a pneumonia which was often fatal.

Symptoms

The onset of the vaccinial reaction was marked by obvious signs of discomfort which generally began on the 3rd day after injection but were occasionally delayed until the 4th day. Often the first indication was a tendency to remain huddled in a corner of the cage. By the 4th day symptoms were usually unmistakable. The mice were thin with a lean hunched appearance and ruffed coats; they were generally inactive and showed a markedly accelerated respiration. A cutaneous reaction was never observed. Nasal irritation was indicated by snuffling and often an intermittent chattering. There was no nasal discharge. The disease usually reached a crisis between the 5th and 7th days, terminating in death or rapid recovery. Occasionally recovery was retarded, symptoms persisting as long as 2 weeks after injection.

Pathology

Postmortem examination on the 3rd to the 7th day showed inflammation of the nasal mucosa but no indication of pock formation. A copious amount of turbid semifluid exudate was often present in the nasal passages. The exudate showed numerous tissue and mononuclear cells; polynuclears were generally present but never predominated as in the exudate of infectious catarrh. Elementary bodies

were detectable by the Morosow stain and were generally present in considerable numbers. An otitis media was sometimes observed but was not a characteristic feature, as it was in mouse catarrh. Bacteria were rarely conspicuous in the nasal exudate, save in mice which had been dead for some time when examined, and coccobacilliform bodies were never seen.

A pneumonia was generally present in infected mice, macroscopic changes being detectable in the lung by the 3rd or 4th day. Involved lobes showed grey or pink to red translucent areas of consolidation. At first these areas were small and patchy, later through coalescence they occupied a considerable portion of the lobe. The pleural surface was often moist, and occasionally free fluid was present in the chest cavity. In the lungs of mice that died it was not unusual to find areas of consolidation in all 5 lobes.

Histologically the vaccinial reaction in the lung was quite unlike any of the native pneumonias that we have seen in mice. The reaction involved both the bronchi and the alveoli. The bronchi frequently showed necrosis which varied in extent from case to case. In some instances there was a central plug of tissue debris. The alveoli were often filled with a coagulated serous deposit, occasionally with red cells. The alveolar walls were usually congested and areas of necrosis were often present. The alveolar reaction was further characterized by the deposition of fibrin as fibrils or dense strands. There was usually a marked increase in mononuclear cells in involved areas. Polynuclear leucocytes were generally present but were rarely numerous. Acidophilic cell inclusions resembling the Guarnieri bodies were not conspicuous. They were never found in the epithelial cells of the bronchi but were sometimes observed, as late as the 5th day, in the cytoplasm of large cells in areas of alveolar involvement. These inclusions were generally multiple, and sharply outlined. They often appeared to be embedded in a clear unstained zone.

The Mortality Rate

The nasal instillation of vaccinia virus was attended by a high though variable mortality, the actual rate being influenced by the mode of injection.

In the earlier experiments the suspension was dropped from a pipette directly in the nasal openings of etherized mice. Later the method used by Shope (8) in his work on influenza in mice was employed. The nose of an etherized mouse was dipped into a thin layer of the suspension which was drawn into the nasal passages with inhalation. Depending on the size of the animal, from 0.1 to 0.2 cc. of fluid was taken up in this way.

As indicated in Table I the percentage mortality was more than doubled in mice infected by dipping, the respective rates being 34 and 72 per cent. This high mortality was also maintained in a second

series of mice infected by dipping. Inoculated in this way a larger volume of suspension was drawn into the nasal passages and was probably drawn deeper. Regardless of the method, however, the mortality rate fluctuated from group to group, ranging from 0 in one group to 100 per cent in 4 groups. These irregularities in mortality were probably referable to variations in dosage. Most of the deaths occurred between the 5th and 7th days after infection. An advanced pneumonia was invariably present and was presumably the cause of death. There was no indication that the mortality was significantly affected by secondary bacteria.

TABLE I

The Mortality Rate in Mice Infected by Injection and by Dipping

Infected by injection			Infected by dipping		
Suspension	Number of mice	Number of deaths	Suspension	Number of mice	Number of deaths
Egg membrane	5	2	Egg membrane	5	4
Exudate	5	0	Exudate	5	1
"	5	0	"	5	0
"	5	2	"	5	5
"	5	3	"	5	4
Egg membrane	5	1	"	5	5
"	5	2	"	5	3
"	5	1	"	5	5
Exudate	5	2	"	5	4
"	5	4	"	5	5
Total.............	50	17	Total.............	50	36

Transmission by Passage

The respiratory disease invoked in mice by vaccinia virus was readily transmitted by passage. Exudate removed from the nasal passages of sick mice at autopsy or from dead mice was regularly infective for normal animals. No significant variations in the disease in respect to symptoms or mortality were noted with continued passage. Two experiments with 10 successive passages were carried out, one being still in progress for the maintenance of infective exudate. The results of the earlier passage are shown in the sixth column of Table I. The suspensions used were approximately a 1:10

dilution of nasal exudate in saline. The morbidity rate of the 50 mice employed in this test was 100 per cent and the mortality rate 72 per cent.

Acquired Immunity

It was not uncommon for mice with severe symptoms, seemingly about to die, to rally suddenly and within several days regain their normal sleek appearance. From time to time mice which had thus recovered were reinjected with an infective suspension of the virus. The interval between the two injections was variable but never less than 2 weeks. Generally the reinfected mice were kept under observation for 10 to 14 days and then autopsied, but in a few instances they were killed within a week of the second injection. 25 mice were tested, with a single non-specific fatality. In no case was there any indication of a reaction to the virus, either during life or at autopsy. Recovery clearly imposed a solid immunity to reinfection by the nasal route. The results with a few mice which were tested after an interval of a year indicated that the immunity does decline. Thus, of 4 reinfected mice, 1 showed a typical vaccinial reaction and died, 1 was normal during life and at autopsy, and 2 had a slight pneumonia when killed.

Communicability by Direct Contact

In each of 5 contact experiments 5 normal mice were placed in the same cage with an equal number of infected mice. The actual period of contact was variable depending on the survival time of the infected animals. None of the 25 exposed mice died and none showed symptoms. A few were sacrificed early with negative findings at autopsy. Most of the exposed mice were reinjected with an infective suspension of vaccinia virus. The susceptibility of the mice in 3 of the groups was normal, the morbidity rate being 100 per cent and the mortality 80 per cent. 2 groups of mice showed definite evidence of protection. There was only one death (10 per cent) and no indication of disease save in this one mouse. In these 2 groups, transmission of the virus was favored by a longer survival of the infected animals.

The outcome of the contact experiments indicated that vaccinial catarrh was not communicable by cohabitation. The amount of virus transmitted from infected to normal mice was regularly below the threshold required to establish an active infection. The actual dissemination of virus during cohabitation was intimated by the acquired resistance of 2 groups of mice in which exposure was prolonged.

The Limiting Infective Dilution of Virus

An infected egg membrane weighing 200 mg. was finely ground and a 10 per cent suspension prepared in saline. Graded dilutions were then made in steps of 10, and 5 mice infected with each dilution. The recorded dilutions which ranged from 2.5×10^{-2} to 2.5×10^{-5} were approximate, as the mice were infected by dipping. The actual variation, however, was slight. 2 embryonated eggs were inoculated with dilutions from 2.5×10^{-2} through 2.5×10^{-6}. They were

TABLE II

The Limiting Infective Dilution of Vaccinia Virus in Mice and in Embryonated Eggs

Mouse test				Egg test		
Dilution	Number of mice	Number showing symptoms	Number of deaths	Dilution	Number of eggs	Reaction
2.5×10^{-2}	5	5	5	2.5×10^{-3}	2	$+$
2.5×10^{-3}	5	5	2	2.5×10^{-4}	2	$+$
2.5×10^{-4}	5	0	1*	2.5×10^{-5}	2	$+$
2.5×10^{-5}	5	0	0	2.5×10^{-6}	2	$+$

* Accidental death, not specific.

TABLE III

Protection in Mice Injected with a Subinfective Dilution of Virus

Group	Number of mice	Number showing symptoms	Number of deaths
Mice injected with dilution 2.5×10^{-4}..............	5	0	0
Survivors injected with undiluted suspension..........	5	0	0
Normal mice injected with " "	5	5	4

opened on the 3rd day after incubation at 37°C. and the membranes were examined for elementary bodies. A protection test was made 4 weeks after injection on mice which showed no reaction to a 2.5×10^{-4} dilution of virus.

The results of these experiments which are recorded in Tables II and III show that the amount of vaccinia virus required to establish infection in the mouse was at least 1000 times greater than that required to infect an embryonated egg and at least 10 times greater

than the immunizing dosage. The end-point was not reached in the eggs, but a dilution of 2.5×10^{-6} was infective. In mice the limiting infective dilution was 2.5×10^{-3} whereas the immunizing dilution was at least 2.5×10^{-4}.

Distribution of the Virus

1. In the Nasal Passages.—The nasal scrapings or exudate from infected mice regularly showed elementary bodies, with the Morosow stain, during the acute stage of the disease. One experiment was made to determine how soon the elementary bodies could be detected after infection.

TABLE IV

The Detection of Virus in the Nasal Passages during the Incubation Period

Time of autopsy	Number of mice	Symptoms	Rhinitis	Elementary bodies	Injection of nasal washings			Protection test		
					Number of mice	Number with symptoms	Number of deaths	Number of mice	Number with symptoms	Number of deaths
hrs.										
24	2	—	—	—	5	—	—	5 survivors	0	1*
48	2	—	—	+	5	5	5	5 normal	5	4
72	2	+	+	+	5	5	5			

* Accidental.

Mice were infected by dipping and killed in groups of 2 at daily intervals through the 3rd day. The nasal scrapings were examined microscopically with the Morosow stain and tested for infectivity, using a 1:10 saline suspension. Survivors of the latter group were tested for protection.

The results of this experiment, as summarized in Table IV, indicate that elementary bodies were present in the nasal passages throughout the incubation period. There was no apparent increase, however, until the 2nd day, and no evidence of a rhinitis until the 3rd day. Additional observations indicate that active multiplication may be delayed until the 3rd day when symptoms are first apparent. Nasal washings removed on the 1st day contained sufficient virus to afford protection in normal mice and on the 2nd day to establish infection.

2. In the Lung.—The numerous granules in the lung tissue films interfered with the examination for elementary bodies, and their presence was never determined with certainty. Infectivity tests, however, indicated that an infective amount of virus was present, at least during the acute stage.   Suspensions made of lung removed from 3 sick mice on the 6th, 6th, and 7th day of the disease produced a characteristic vaccinial reaction in each of 15 normal animals. The mortality rate was 55 per cent.   Elementary bodies were again

TABLE V

The Detection of Vaccinia Virus in the Blood by Culture in Embryonated Eggs

Blood sample		Reaction		
Number	Day drawn	Lung	Nasal passage*	Egg
1	2nd	−	−	+
2	2nd	−	−	+
3	2nd	−	−	+
4	3rd	−	+	+
5	3rd	−	+	+
6	3rd	−	+	+
7	4th	−	+	+
8	4th	−	+	+
9	5th	+	+	+
10	5th	+	+	+
11	6th	+	+	+
12	6th	+	+	−
13	6th	+	+	−
14	6th	+	+	−
15	7th	+	+	−

* Includes presence of elementary bodies.

demonstrable in the nasal exudate at autopsy.   A suspension made of lung removed on the 14th day from a mouse with persistent symptoms was not infective for 5 normal mice but did protect them against active nasal exudate.

3. In the Blood.—

Mice infected by dipping were bled from the heart under deep ether anesthesia. To avoid possible contact with the lung the chest cavity was exposed prior to bleeding.   The aspirated blood, which varied from 0.2 to 0.5 cc. in volume, was made up to 1.0 cc. with saline and approximately 0.1 cc. of the suspension was implanted on the chorio-allantoic membrane of an embryonated egg.   The inocu-

lated eggs were incubated at 37°C. for 3 days and opened. Membranes which showed a visible reaction were examined for elementary bodies.

As shown in Table V, 11 of the 15 samples of blood contained sufficient virus to produce egg membrane lesions. The reaction was confined to discrete foci of varying size, in which elementary bodies were generally demonstrable. The number of foci varied from 5 to 50 per membrane, the latter indicating a virus content in the blood of at least 500 elementary bodies per cubic centimeter. None of the eggs showed a diffuse necrotic reaction or death of the embryo indicative of a high virus concentration. Virus was detectable in the blood as early as the 2nd day, prior to the appearance either of a rhinitis or a pneumonia. 4 of the 5 samples removed on the 6th or 7th day contained no virus.

4. *In the Skin.*—Development of the virus in the skin following nasal instillation was never observed. The strain employed does not produce the characteristic pox of vaccinia even when introduced directly into the skin. It does, however, produce a superficial necrosis with considerable reaction in the subcutaneous tissue, followed by scab formation but no scar. Since the virus was generally demonstrable in the blood, it was thought that injury to the skin prior to nasal instillation might induce cutaneous localization of the virus. 6 mice were shaved over the abdomen and infected by dipping. 4 died on the 4th to the 6th day with no skin lesions. 2 mice which recovered also showed no evidence of a cutaneous reaction.

DISCUSSION

Vaccinia virus implanted on the untraumatized nasal mucosa of mice multiplies actively in the local epithelial cells. After a lag of several days, following the introduction of the virus, elementary bodies are demonstrable microscopically and exudation is apparent. The nasal surfaces are evidently highly permeable to the virus as it is cultivable from the blood prior to its microscopic appearance in the nasal tract. From its point of entrance the virus may be carried to the lung by two routes: directly, by way of the blood, and indirectly, along the bronchi. There is no apparent cutaneous development of the virus although it is probably carried to the skin in the circulating blood.

The behavior of vaccinia virus administered to mice by nasal instillation resembles that in rabbits similarly infected. In both hosts there is a catarrhal reaction with a coryza and pneumonia. These two manifestations are almost invariably associated in mice. In rabbits, however, the reported observations indicate that they are generally not associated, one occurring in the absence of the other. If these observations are indicative of strain differences either in the host or the virus, similar irregularities may be expected with other vaccinia strains in mice.

The factor of dosage is important in establishing vaccinia virus in the respiratory tract of mice. With the present strain the amount required to infect a mouse by the nasal route was at least 1000 times that required to infect an embryonated egg. The importance of dosage was also indicated by the failure to establish a vaccinial catarrh by contact infection. In the rabbit, however, vaccinia or vaccinia-like viruses may be naturally transmitted by the nasal passages and result in outbreaks of epidemic proportions. It appears probable that the mouse is endowed with a greater natural immunity to vaccinia than is the rabbit and is susceptible only when a critical concentration of virus is introduced.

The general manifestations of vaccinial catarrh in the mouse resemble those of the catarrh produced by the coccobacilliform bodies. Both show similar symptoms and both are characterized by a coryza and pneumonia. In specific details, however, the two reactions are quite unlike. Infectious catarrh is of slow onset and chronic. The disease progresses slowly over an extended period and is invariably fatal. The inflammatory reaction in all involved loci is characterized by a predominance of polynuclear leucocytes. The disease is readily transmissible by direct contact, and the specific agent is demonstrable in the nasal passages throughout the life of the host. Vaccinial catarrh is of rapid onset. The course of the disease is short and is abruptly terminated by death or rapid recovery. Recovered mice are immune to reinfection for a considerable period of time. The inflammatory reaction is characterized by a predominance of mononuclear cells. Vaccinial catarrh is not communicable by cohabitation, but sufficient virus may be disseminated thereby to produce immunity in exposed individuals.

SUMMARY

A catarrhal reaction manifested by a coryza and a pneumonia of characteristic pathology was regularly produced in mice by the nasal instillation of vaccinia virus. Inoculation into embryonated eggs indicated that the virus entered the circulation as early as the 2nd day after injection.

The vaccinial catarrh was readily transmissible by the passage of nasal exudate but not by contact. Dosage was important in establishing the virus in the nasal passages, the limiting dilution being approximately 10^{-3} of an egg membrane suspension (at least 1000 times the amount required to infect an embryonated egg).

The morbidity rate was variable but in general high, reaching 70 per cent in 2 groups of 50 mice. An immunity which was effective against reinfection for several months but ultimately declined was attendant on recovery. The amount of virus required to produce this immunity was significantly less than the infective dosage.

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EXPLANATION OF PLATE 12

FIG. 1. Elementary bodies in nasal exudate. Morosow stain. × 1000, enlarged to 3000.

FIG. 2. Vaccinial foci in egg membrane inoculated with blood. Approximately natural size.

FIG. 3. Late vaccinial reaction in lung. Phloxin methylene blue stain. × 125.

FIG. 4. Alveolar cell with multiple cytoplasmic inclusions. Phloxin methylene blue stain. × 1200.

FIG. 5. Alveolar reaction showing strands of fibrin. Phloxin methylene blue stain. × 800.

PLATE 12
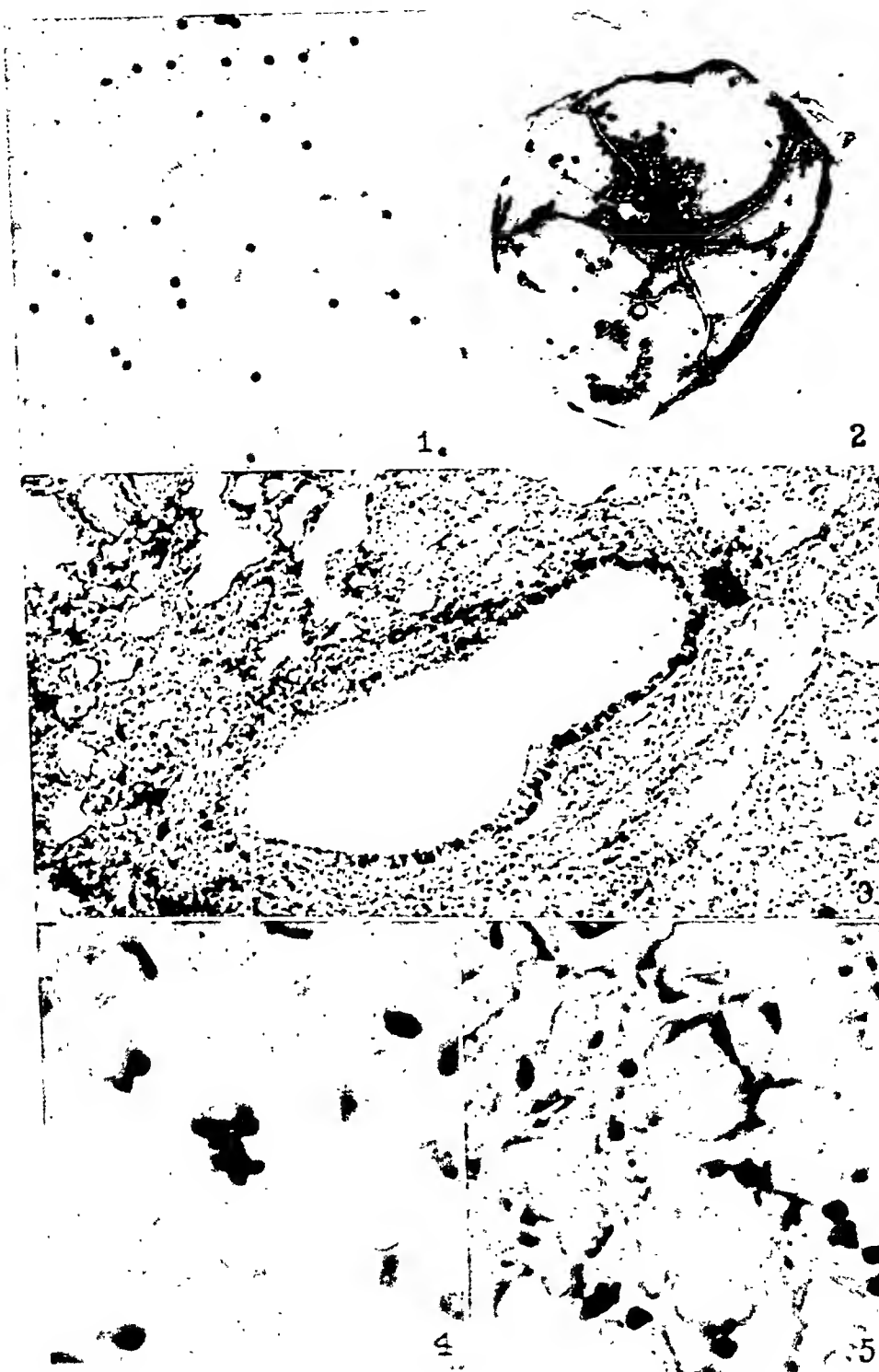
MOLECULAR WEIGHT, ELECTROCHEMICAL AND BIOLOGICAL PROPERTIES OF TUBERCULIN PROTEIN AND POLYSACCHARIDE MOLECULES*

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PLATE 13

It is of fundamental importance to obtain as nearly complete an understanding as possible of the size and other physical properties of the molecule or molecules in tuberculin which are able to elicit the tuberculin reaction. Not only is such knowledge of great value in the preparation of a pure product and in further attempts to explain the cause of the tuberculin reaction itself and its relationship to the disease, but also it will be helpful in interpreting a whole series of similar immunological reactions of which the tuberculin reaction is a prototype. The differences observed between the tuberculin reaction (the delayed type of reaction) and typical protein sensitization reactions (the immediate type) may well depend upon some physical property of the molecule, for both are obviously elicited by constituents in the tubercle bacillus culture filtrates.

Throughout this study, therefore, the relationship in various preparations between tuberculin potency and antigenicity, on the one hand, and molecular weight or shape and electrochemical properties, on the other, was noted, and the aim was to prepare homogeneous fractions having certain of these different properties. The separations were rendered difficult because of the large amount of polysaccharide

and in some cases of nucleic acid present with the protein. Neither of these diluting components, however, seemed to be responsible for any of the physiological reactions noted and obtained when the fractions were injected. The polysaccharide has been studied in considerable detail in order to understand its relationship to the protein and methods for its elimination.

Methods

Studies of the sedimentation velocities in the Svedberg ultracentrifuge (1), rotating at a velocity of 60,000 to 70,000 R.P.M. (centrifugal force of 285,000 to 360,000 times gravity) were made on the various tuberculin preparations. The diffusion constants of those fractions which appeared to be homogeneous in the centrifuge were then determined by the method developed by Lamm (2), using the cell designed by him. The refractive index scale method was used in both centrifuge and diffusion procedures. Specific volume determinations were also made on representative fractions. All sedimentation constants reported in this paper have been expressed in units of 10^{-13}, and diffusion constants in units of 10^{-7}.

The sedimentation constant was calculated from the formula given by Svedberg (3)

$$s_{20} = dx/dt \cdot 1/\omega^2 x \cdot \eta/\eta_0 \cdot \frac{1 - V\rho_0}{1 - V\rho},$$

where dx/dt = the sedimentation velocity observed, ω = the angular velocity, η/η_0 = the ratio of the viscosity of the solution to that of water at 20°C., V = the partial specific volume of solute, and ρ and ρ_0 the densities of solution and of water at 20°C. A brief description of the technique involved has been given recently (4).

The diffusion constant was calculated as an average of the constants determined for seven or eight points on each curve of four to six exposures taken during the diffusion, using the formula (equation 4) in the paper of Lamm and Polson (5).

From the diffusion constant thus obtained and the sedimentation constant found on the same solution the molecular weight may be calculated according to the formula given by Svedberg (6),

$$M = \frac{RT_s}{(1 - V\rho)D},$$

where R is the gas constant, T the absolute temperature, and ρ the density of the solution.

The molar frictional constant, f, of the molecule may also be calculated from these data, thus:

$$f = RT/D = \frac{M(1 - V\rho)}{s}.$$

If, however, the molecule is compactly spherical and unhydrated the following formula,

$$f_0 = 6\pi\eta N \left(\frac{3MV}{4\pi N}\right)^{\frac{1}{3}},$$

where N is the Avogadro number, will be valid.   For such a molecule, therefore, the frictional ratio f/f_0 should be unity.   A larger value of f/f_0 indicates that the molecule either deviates from the compact spherical shape or that it is hydrated.

The degree of molecular weight homogeneity of a fraction could be judged to some extent by observing the symmetry of the sedimentation curves made from exposures taken at different intervals during a run, and also by noting the agreement between the D_{20} constants determined for a number of points on the diffusion curves made from various exposures.   In several cases a further criterion of the homogeneity of a fraction was gained by comparing the D_{20} obtained from a real diffusion experiment with the D_{20} calculated from the sedimentation curves. A small degree of heterogeneity would reveal itself as a marked deviation between the two and in such case the diffusion constant computed from the sedimentation diagram would be only an apparent one.

The concentration of the sedimenting molecules may be calculated from the sedimentation diagrams, certain constants for the apparatus, and the refractive index increment for the solution (4, 7).   The refractive index increment of a fraction which was practically homogeneous in the centrifuge (TPA-21b, see later), was measured in the Pulfrich refractometer and found to be 0.001948 at wave length $\lambda = 579$ mμ.

Electrochemical homogeneity was determined by means of electrophoresis in the Tiselius apparatus (9).   There is, however, a noteworthy difference to be considered between electrochemical homogeneity and molecular weight homogeneity as determined by sedimentation and diffusion.   Earlier investigations (10) have given examples of cases of homogeneous sedimentation and diffusion but inhomogeneous electrophoresis (e.g. the serum globulins), on the one hand, and of inhomogeneous sedimentation and diffusion but homogeneous electrophoresis (e.g. dissociation of proteins into particles of nearly unchanged chemical nature) on the other.   In this investigation the criterion for electrophoretic homogeneity was limited to the number of individual boundaries migrating, as representative of separate components, and not to the purity of the individual boundaries.

Chemical analyses were made upon most of the fractions as follows: Total concentration of a solution was determined by means of the dipping refractometer. Nitrogen determinations were made by means of a modification of the micro Kjeldahl method of Pregl.   Carbohydrate was determined at first by the orcin-sulfuric acid method of Tillmans and Philippi (11), but later better results were obtained using the carbazol reaction of Dische (12).   The tuberculin polysaccharide prepared by one of the authors and described later, was used as a standard, and by means of the shade of color obtained in this carbazol reaction it was possible to distinguish immediately between the presence of real tuberculin polysaccharide

which gave a slightly brownish pink color because of the mannose present (13), and that of nucleic acid carbohydrate which gave a reddish pink color. Standards containing 0.1, 0.2, and 0.3 mg. polysaccharide and blank determinations were made with each set of analyses.

Nucleic acid was determined by means of the diphenylamine reaction of Dische (12), using a very highly purified preparation of thymus nucleic acid[1] as standard, since the nucleic acid of the tubercle bacillus is known to contain thymine (14). A blank and standards containing 0.1, 0.2, and 0.3 mg. nucleic acid were run with each set of analyses. The reaction was highly specific for the tubercle bacillus nucleic acid when the proportion of reagents recommended by Dische were used (1 gm. diphenylamine in 100 cc. glacial acid plus 2.74 cc. concentrated sulfuric acid. Two parts of this reagent and one part of the unknown solution were heated in a boiling water bath for 10 minutes). The tuberculin polysaccharide gave no reaction.

The biological potency of the fractions was tested by means of intracutaneous reactions with 0.1 cc. amounts on tuberculous guinea pigs. The reactions were measured in three dimensions 24 and 48 hours after the injection, and these readings were then averaged in all of the animals tested. Toxicity tests for tuberculous guinea pigs were made by intraperitoneal injections. In those cases where the product was lethal the animal was dead within 24 hours and at autopsy showed the pathological changes of typical tuberculin death, namely, inflation of the lungs and fluid, fibrin and congestion in the splanchnic area. Precipitin tests for the protein fractions were made with rabbit antiserum[2] to the whole mixed protein fraction from tuberculin, containing a very low content of polysaccharide, and for polysaccharide with the anti-tubercle bacillus horse serum.[3]

Two groups of materials were used in these experiments. The first consisted of the unchanged culture medium filtrates, where the molecules would be found in their most natural state. The second group included OT (old tuberculin) which had been heated during its preparation and which would, therefore, be expected to contain molecules markedly changed from their natural state. This substance, as well as fractions isolated from it, such as the purified protein derivative of tuberculin (hereafter designated as PPD) (15), are of especial interest because of their practical value in medical practice.

<center>EXPERIMENTAL</center>

Original Tuberculin Culture Medium Filtrates.—The first studies were made on the unchanged culture medium filtrates produced by

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[1] This preparation of nucleic acid was kindly given to us by Professor E. Hammarsten, Stockholm.

[2] We are indebted to Dr. P. Kallós for facilities and assistance in obtaining this serum.

[3] Prepared by the Mulford Laboratories of Sharp and Dohme, and sent to us by Dr. Michael Heidelberger.

growing two human strains (PN and H 37) and one bovine (523) strain of tubercle bacilli and the Moeller strain of timothy grass bacillus on the Long synthetic medium for 8 weeks, filtering off the bacilli through the Seitz filter, concentrating this filtrate on the ultrafilter, and washing it until it was salt-free. These fractions were called TPU, prefixed to the name of the strain, meaning tuberculin protein ultrafiltered.

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TPU-human (PN)-unpreserved.........................	1.7		
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+ merthiolate (1:10,000)...........	1.8	35.7	0.54
TPU-human (H 37)................................	1.8	72.5	1.10
TPU-bovine (523)................................	1.7	32.1	0.26
TPU-timothy (Moeller)............................	1.4	26.8	0.49
	1.6		

symmetrical throughout the entire run. The s_{20} determinations on the TPU-human (PN) fractions were made at pH 6.8; all the others were made at pH 8.0. In all cases phosphate buffer plus 0.2 M NaCl, giving a total molarity of 0.235–0.25, was used.

The three determinations upon TPU-PN, namely, unpreserved, preserved with 0.5 per cent phenol, or merthiolate[4] (1:10,000), indicated that very little change was caused by the preservatives used, except that slightly more of the quickly sedimenting components (probably denatured protein) were present. Carbohydrate analyses showed the presence of considerable polysaccharide in all cases.

These fractions were studied in the Tiselius electrophoresis apparatus at 0°C. in the presence of phosphate buffer at pH 8.0 and of

[4] The merthiolate was obtained from Eli Lilly and Company.

which gave a slightly brownish pink color because of the mannose present (13), and that of nucleic acid carbohydrate which gave a reddish pink color. Standards containing 0.1, 0.2, and 0.3 mg. polysaccharide and blank determinations were made with each set of analyses.

Nucleic acid was determined by means of the diphenylamine reaction of Dische (12), using a very highly purified preparation of thymus nucleic acid[1] as standard, since the nucleic acid of the tubercle bacillus is known to contain thymine (14). A blank and standards containing 0.1, 0.2, and 0.3 mg. nucleic acid were run with each set of analyses.   The reaction was highly specific for the tubercle bacillus nucleic acid when the proportion of reagents recommended by Dische were used (1 gm. diphenylamine in 100 cc. glacial acid plus 2.74 cc. concentrated sulfuric acid.   Two parts of this reagent and one part of the unknown solution were heated in a boiling water bath for 10 minutes).   The tuberculin polysaccharide gave no reaction.

The biological potency of the fractions was tested by means of intracutaneous reactions with 0.1 cc. amounts on tuberculous guinea pigs.   The reactions were measured in three dimensions 24 and 48 hours after the injection, and these readings were then averaged in all of the animals tested.   Toxicity tests for tuberculous guinea pigs were made by intraperitoneal injections.   In those cases where the product was lethal the animal was dead within 24 hours and at autopsy showed the pathological changes of typical tuberculin death, namely, inflation of the lungs and fluid, fibrin and congestion in the splanchnic area.   Precipitin tests for the protein fractions were made with rabbit antiserum[2] to the whole mixed protein fraction from tuberculin, containing a very low content of polysaccharide, and for polysaccharide with the anti-tubercle bacillus horse serum.[3]

Two groups of materials were used in these experiments.   The first consisted of the unchanged culture medium filtrates, where the molecules would be found in their most natural state.   The second group included OT (old tuberculin) which had been heated during its preparation and which would, therefore, be expected to contain molecules markedly changed from their natural state.   This substance, as well as fractions isolated from it, such as the purified protein derivative of tuberculin (hereafter designated as PPD) (15), are of especial interest because of their practical value in medical practice.

<div align="center">EXPERIMENTAL</div>

Original Tuberculin Culture Medium Filtrates.—The first studies were made on the unchanged culture medium filtrates produced by

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[1] This preparation of nucleic acid was kindly given to us by Professor E. Hammarsten, Stockholm.

[2] We are indebted to Dr. P. Kallós for facilities and assistance in obtaining this serum.

[3] Prepared by the Mulford Laboratories of Sharp and Dohme, and sent to us by Dr. Michael Heidelberger.

growing two human strains (PN and H 37) and one bovine (523) strain of tubercle bacilli and the Moeller strain of timothy grass bacillus on the Long synthetic medium for 8 weeks, filtering off the bacilli through the Seitz filter, concentrating this filtrate on the ultrafilter, and washing it until it was salt-free. These fractions were called TPU, prefixed to the name of the strain, meaning tuberculin protein ultrafiltered.

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ionic concentration 0.1 or 0.2, with the corresponding appropriate voltages of 300 or 200. The TPU-human (PN) was found to have three boundaries migrating toward the positive pole. The fastest and slowest of these boundaries were thin, while the intermediate one was very heavy. In the cases of TPU-bovine and TPU-timothy only two mobile boundaries appeared, but both were equally heavy. In all preparations an extra very heavy immobile band was present, which proved to be caused by polysaccharide. Since the mobilities of the protein and polysaccharide components were so different electrophoresis seemed a promising method for separating them.
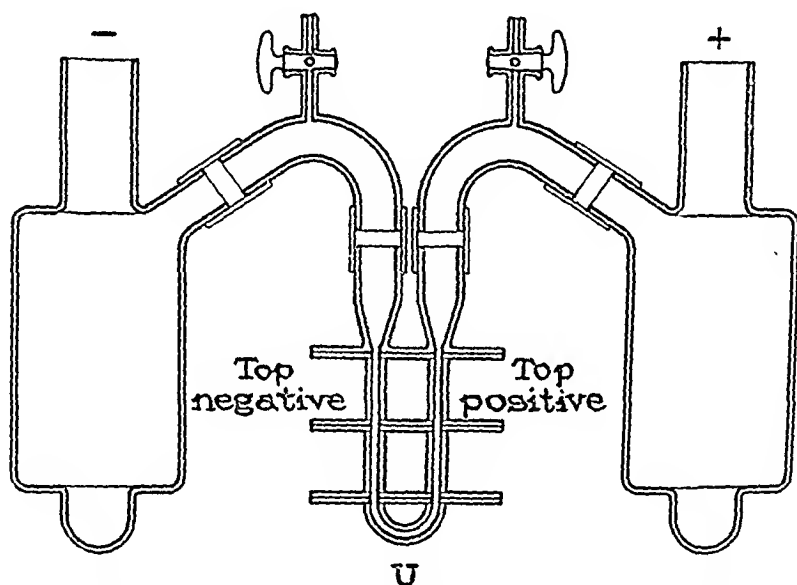
TEXT-FIG. 1. Diagram of Tiselius electrophoresis apparatus.

Fractions Separated by Electrophoresis.—The experiments were so arranged that the current was allowed to flow until the top positive compartment (see Text-fig. 1) was filled with the solution exhibiting the two mobile components. By compensation in the opposite direction it was possible to move the carbohydrate boundary down into the U tube and thus to fill the two compartments on the positive side with the protein solution. This solution, from these two compartments on analysis was found to contain a much lower percentage of carbohydrate than the original solution and correspondingly higher content of protein. It was separated from the remainder of the

solution and run again in the apparatus in the same way, using fresh buffer, except in the case of TPU-timothy. In this manner, it was possible to obtain fractions with very low carbohydrate content (see Table II).

In the final TPU-human and timothy fractions two components were, therefore, still present, as shown by the presence of two electrophoretic boundaries. When run in the centrifuge the TPU-human (PN) showed the presence of two clearly defined components with $s_{20} = 1.9$ and 4.9 in two different experiments 1 and 2, as shown in

TABLE II

Fractions Separated by Electrophoresis

Tuberculin (TPU) used	Per cent of total as carbohydrate	Sedimentation data						Mobilities of boundaries in top positive compartment (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)
		pH	Buffer concentration	α-component		β-component		
				s_{20}	Per cent concentration*	s_{20}	Per cent concentration*	
			μ					
Human (PN)								
Experiment 1......		7.0	0.05	1.9	0.28	4.9	0.095	
Experiment 2......	3.6	7.0	0.05	2.0	0.47	4.9	0.22	7.8
								5.3
Bovine (523)........	4.7	8.0	0.235	1.6	0.42			6.6
Timothy (Moeller)....	9.4	8.0	0.235	1.4				{ 11.5 ?

* The concentrations were calculated from the area of the sedimentation curves according to the formula given under methods.

Table II. The larger molecule does not seem to appear in the original medium and may, therefore, have been formed during the separation of protein and polysaccharide. The TPU-timothy, however, gave on sedimentation only one main gradient, but it was markedly heterogeneous.

In the case of the TPU-bovine, during the final separation, it was possible, by running for a considerable length of time, to separate the two boundaries a half cell distance and, therefore by pipetting out the upper half, to obtain about 2 cc. of a solution with only one electrophoretic component. This was sufficient for centrifuge and diffusion

runs and analyses.   It proved to be a single homogeneous substance in the centrifuge and also in diffusion, with constants of $s_{20} = 1.6$ and $D_{20} = 12.0$ respectively.   Assuming a specific volume of 0.700 (the same as found for the human strain protein), a molecular weight of about 10,000 was calculated.   See Table X.

Biological tests were made upon some of the fractions separated from the TPU-human (PN).   The summary of the skin reactions in tuberculous guinea pigs and of the precipitin tests is given in Table III.   The protein fraction was that fraction migrating into the top positive compartment and described in Table II.   The carbohydrate-protein fraction was that which remained behind on the negative

TABLE III

Biological Potency of Fractions Separated by Electrophoresis

Substance	Per cent carbohy-drate	Average dimensions of skin reactions (Five tuberculous animals each)			Precipitin with least amount protein
		First set 0.005 mg. solid	Second set 0.005 mg. protein	Third set 0.005 mg. protein	
		mm.	*mm.*	*mm.*	*mg.*
Protein	3.6	13 x 14 x 1.7	12 x 12 x 1.3	10 x 10 x 1.3	0.0008
Carbohydrate-pro-tein	40.0	13 x 14 x 1.7	15 x 17 x 1.9		0.0012
Carbohydrate	85.6	4 x 4 x 0.4	2 x 2 x 0.25		0.0011
Standard PPD	24.0	13 x 14 x 1.6	17 x 13 x 1.4	9 x 11 x 1.3	
TPU-human (PN)	35.0			15 x 18 x 2.4	

side of the U tube with the polysaccharide and, therefore, was probably the fraction with slowest migration and most closely associated with the polysaccharide.   The carbohydrate fraction was obtained by electrophoresis as described in the section under polysaccharide.

Table III shows that the tuberculin protein separated from the carbohydrate by electrophoresis had about the same potency as the standard PPD which has been used in extensive tuberculin testing (15).   Furthermore, the protein which was most closely associated with the carbohydrate was probably somewhat more potent per unit of protein than the free protein itself and slightly less antigenic, as demonstrated by the precipitin tests.   Toxicity tests on tuberculous animals also suggested (Table IV) that the free protein may be less

toxic than that protein associated with carbohydrate. In Table IV the numbers in circles indicate the animals which died from the injections. That the increased toxicity was not due to a toxic carbohydrate is indicated by the fact that even as much as 10 mg. of the polysaccharide, isolated in this way in its most nearly native form, was not toxic. Previous studies on the polysaccharide isolated in quantity by chemical means showed that even as much as 50 mg. given intraperitoneally would not kill tuberculous guinea pigs. Probably, therefore, the protein fraction most closely associated with the carbohydrate may be somewhat more potent than the separated protein, but the differences observed are only suggestive and should be confirmed before they can be considered really significant.

TABLE IV

Lethal Potencies

Electrophoretic fractions	Number of animals injected and number killed by the following doses in terms of mg. protein injected						
	10 mg.	3 mg.	2 mg.	1.5 mg.	1 mg.	0.7 mg.	0.5 mg.
TPU-human (PN)...............			①	1	1		1
Protein.........................			2	3	①*		1
Carbohydrate-protein..............			①	①	①	1	
Carbohydrate....................	1	1					1

* This animal must have died of complications since five others receiving more did not die.

Tuberculin Polysaccharide.—The polysaccharide was isolated in electrophoresis by pushing the immobile component into the top compartment on the negative side by the compensation process, and allowing the protein to migrate away from it, and then re-running in the same way the solution from the two compartments on the negative side. The final solution in the top negative compartment had a total concentration of 0.64 per cent, all of which proved to be polysaccharide on analysis. The next lower compartment contained a solution with total concentration 1.07 per cent, of which 90 per cent was polysaccharide and about 7.5 per cent protein. This fraction was analyzed in the centrifuge in two different experiments and in both was found to have one main gradient, with $s_{20} = 1.76$ and 1.81, and a very small amount of a more quickly sedimenting substance, probably protein.

This polysaccharide was then compared with one isolated in quantity in 1931 by the method described by Renfrew (13) and modified by Masucci, McAlpine, and Glenn (16). The latter contained only 0.09 per cent nitrogen and was completely soluble in water, giving a colorless solution. It caused no skin reaction in tuberculous guinea pigs and also had no toxicity, as mentioned above. When studied in the centrifuge in concentrations between 0.2 and 2.0 per cent in phosphate buffer at pH 8 containing 0.2 molar sodium chloride and a total molarity of 0.235, it showed a single homogeneous component. The values for the sedimentation constant showed no drift with change in the concentration but fluctuated between 1.6 and 1.9, around the mean $s_{20} = 1.75$. It was, furthermore, practically homogeneous when tested in diffusion, with a constant of $D_{20} = 11.0$. The specific volume at 20°C. was found to be 0.619, and from these data a molecular weight of 9000 was calculated (see Table X). Apparently the tuberculin polysaccharide differs remarkably from polysaccharides hitherto studied[5] in exhibiting molecular homogeneity.

This latter preparation, when compared with the one isolated by electrophoresis, was found to have the same precipitating power for anti-tubercle bacilli serum, both giving a definite precipitate in a final antigen dilution of 1:6,000,000. It is significant that the sedimentation constant of the polysaccharide is so nearly like that of the main protein component in the tuberculin from the human type tubercle bacillus. Therefore, in the sedimentation graph of the original culture the curves for the two would be practically superimposed upon one another, presenting one curve that may give the false impression of a single homogeneous substance.

Fractions Separated by Chemical Procedures from the Culture Filtrates of Human Tubercle and Timothy Strains of Bacilli.—Fractions isolated from the human type tubercle bacillus (H 37) culture filtrate by trichloroacetic acid precipitation (17), by repeated half or complete saturation with ammonium sulfate (18), by electrodialysis of the half-saturated fraction, by fractional ultrafiltration (18), and by chromatographic adsorption (19) to aluminum hydroxide and elution at pH 8,

[5] Recent investigations (Svedberg and Gralén) on plant juices show that the polysaccharides of native liquids are as a rule homogeneous (private communication from Professor Svedberg on unpublished results).

all gave asymmetrical curves when studied in the ultracentrifuge, indicating more or less molecular heterogeneity.

However, during these studies it was found that a considerable amount of an extremely polydisperse denatured fraction precipitated when the original culture medium or the half-saturated ammonium sulfate fractions were brought to pH 4.6–4.8 with acetate buffer. Therefore, this fraction was removed, and then it was possible to obtain a small amount of a fraction, which was practically homogeneous in the ultracentrifuge and also in diffusion, by one-tenth to one-fourth saturation with ammonium sulfate at pH 4.8. It was

TABLE V

Fractions Made by Ammonium Sulfate Precipitation

Substance	Per cent carbohydrate	Per cent concentration of solution	Buffer concentration			pH	s_{20}	D_{20}	V	Molecular weight
			NaCl	Na₂HPO₄	NaH₂PO₄					
			μ	μ	μ					
TPA-30b	3.1	0.87	0.2	0.033	0.002	8.0	3.3	8.2		32,000
		1.14		0.033	0.002	8.0	3.0*	8.2*		
TPA-30K	2.0	1.25		0.033	0.002	8.0	3.0*	8.4*	0.7	
TPA-21b	4.4	0.65		0.0066	0.0001	8.0	2.9*	8.8*		
TPA-21h	5.4	1.00		0.033	0.002	8.0	3.0*	8.7*		
TTPA-A		1.04	0.04	μ-acetic		4.8	1.8	10.2	0.748	17,000
			0.06	μ-Na acetate						

* Molecular weights were not calculated from these data because of a possible charge effect.

necessary to repeat the precipitation at least four times. Four preparations were made in this way (see Table V). The TPA-30b gave the most ideal curves in the centrifuge, and since the buffer concentration (0.235 molar) was sufficient to eliminate all possible Donnan effect, the molecular weight calculated from these constants, 32,000, is probably the most accurate figure.

The TPA-30K, while not quite so homogeneous in the centrifuge, was studied further, since it was the only fraction obtained in sufficient quantity; about 28 cc. of a 1.25 per cent solution was obtained. It was found to have 16.3 per cent nitrogen, based on the dry weight,
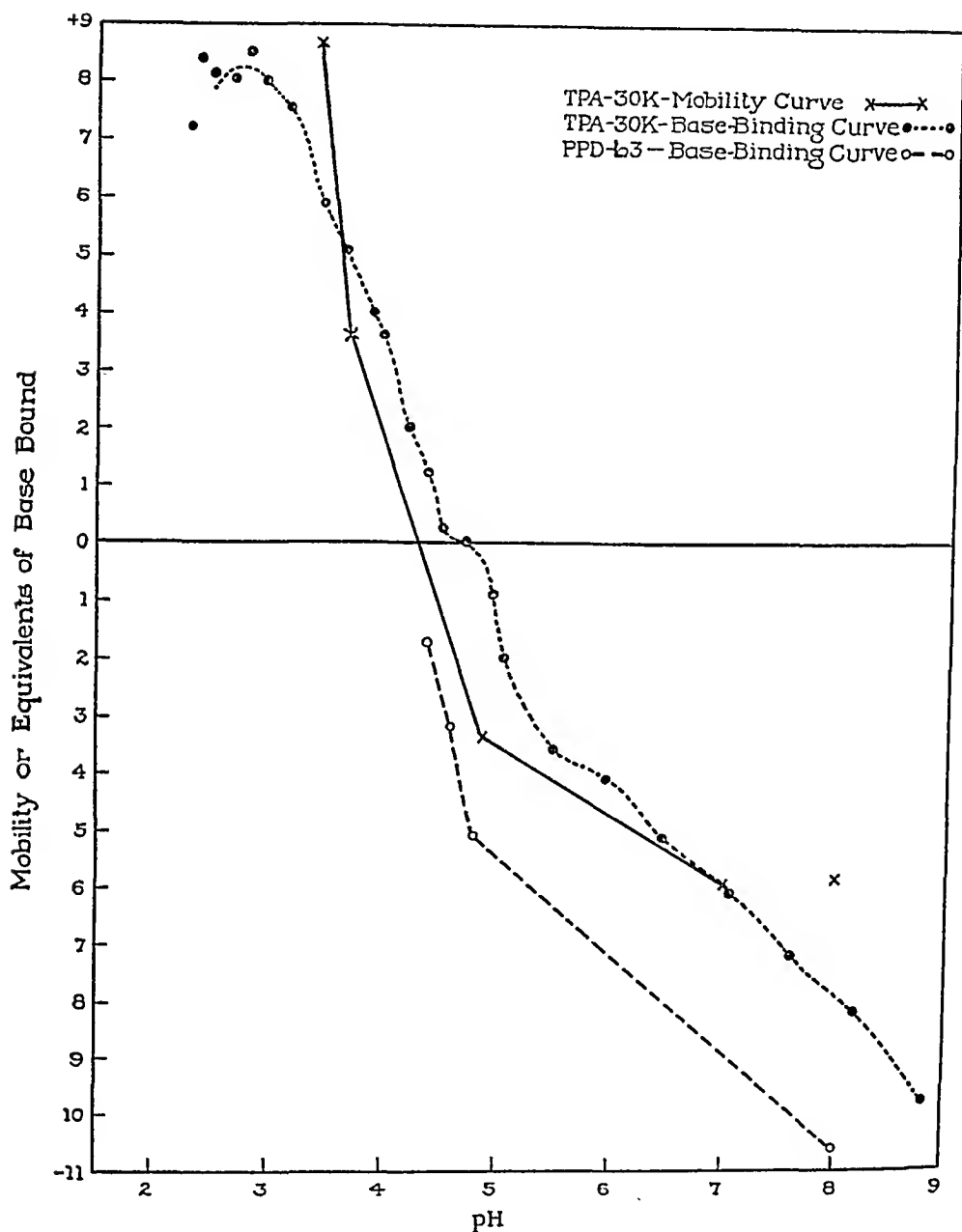
TEXT-FIG. 2. Isoelectric and isoionic points of TPA-30K and base-binding curves of TPA-30K and PPD-b3.

and 2 per cent polysaccharide. After being either frozen at $-8°$ to $-10°$C. or dried *in vacuo* with its buffer at pH 8, there was no evidence of alteration in its sedimentation characteristics.

When studied in electrophoresis it showed a single boundary. By rewashing and reconcentrating the solution on the ultrafilter between each determination with different buffers of $\mu = 0.03$, it was possible to obtain the migration velocities at different pH's, as indicated in the curves (Text-fig. 2, heavy line), and thus to determine the isoelectric point, which proved to be at pH 4.3.

Furthermore, a portion of the solution was washed free of buffer on the ultrafilter and then electrodialyzed. A small amount of a precipitate formed. This was filtered off and then the solution was titrated[6] electrochemically, using the glass electrode. A blank titration in water was first made by adding 0.25 cc. one-tenth normal hydrochloride acid in a total volume of 2.25 cc. and then titrating back with small additions of one-tenth normal sodium hydroxide. Thus the correction to be added to the hydrogen ion concentration at every volume increment was determined. Similar titration was then made with the protein solution, and, using the blank corrections determined, the equivalents of base bound at each pH were calculated as described in a previous publication (20). In this way the isoionic point was found to be at pH 4.7. (See Text-fig. 2, dotted line.) When the pH 4.3 was reached during the titration, a sharp and heavy isoelectric precipitate occurred, which again disappeared as this point was passed. This difference noted between the isoelectric and isoionic points is in accord with the finding of Tiselius (9) on egg albumin and, furthermore, Adair and Adair (21) reported a difference of 0.4 pH in their studies on hemoglobin.

Only 0.0009 mg. of this TPA-30K fraction was required to give a definite precipitate with 0.1 cc. rabbit antiserum to the whole mixed protein fraction.

When tested intracutaneously in tuberculous guinea pigs a surprising demonstration of toxicity and of the local anaphylactic type of reaction was obtained. One normal and nine tuberculous guinea pigs were given simultaneous tests with 0.05 mg. doses of several fractions, among which was the TPA-30K, representing the large molecule, and PPD-b3, which will be described later and which represented the small molecule (see Table VI). The total dosage of

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[6] We are indebted to Dr. Torsten Teorell for kindly making this titration.

all the fractions injected was only 0.25 mg. of which 0.15 mg. was comparatively inactive material. Nevertheless four of the nine guinea pigs were dead at 24 hours and one more died at 48 hours. Therefore, this substance was much more effective than any product previously studied in producing death in the tuberculous guinea pig, since the usual lethal dose of tuberculin protein is about 1 mg. All of those guinea pigs surviving for 24 hours showed very large edematous, diffuse, and deep reddish or purple local reactions at the site of the TPA-30K injections, in contrast to the comparatively small

TABLE VI

Dimensions of Skin Reactions in Tuberculous Guinea Pigs

Guinea pig No.	TPA-30 K (0.05 mg.)		PPD-b 3 (0.05 mg.)	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.
	mm.	*mm.*	*mm.*	*mm.*
Normal	0	0	0	0
TB 1	30 x 35 x 4 (purple)	15 x 25 x 3	15 x 20 x 2	28 x 20 x 5
TB 2	30 x 22 x 3	13 x 15 x 2	7 x 7 x 1	21 x 21 x 3
TB 3	30 x 22 x 3 (red)	12 x 20 x 3	20 x 11 x 2	25 x 18 x 3 (center)
TB 4	12 x 13 x 2 (purple)	12 x 12 x 2	?	25 x 30 x 4 (center)
TB 5	20 x 30 x 3 (purple)	Dead	?	
TB 6	Dead		.	
TB 7	"			
TB 8	"			
TB 9	"			

and more localized or typical tuberculin reactions, at the site of the PPD-b3 injections. At 48 hours the relative sizes of these two reactions in each guinea pig were reversed and the typical delayed reaction with PPD-b3 was the larger of the two. Moreover, the edematous and reddish or purple character of the TPA-30K reactions had disappeared.

Fractionation of the Protein from the Timothy Bacillus.—Since one-tenth to one-fourth saturation with ammonium sulfate yielded an homogeneous fraction from the human type tubercle bacillus

tuberculin an attempt was made to secure a similar fraction from the timothy type bacillus filtrate. The result was complete failure, however, for when tested in the centrifuge the fraction was far from homogeneous. It was, therefore, redissolved in citrate buffer at pH 3 and reprecipitated three times by 0.25 saturation with ammonium sulfate and then washed on the ultrafilter with acetate buffer at pH 4.8. This fraction appeared practically homogeneous in the centrifuge and diffusion and, with the determination of the specific volume, as recorded in Table V, it was possible to calculate a molecular weight of 17,000. (See also Table X.) It is probable, therefore, that the original molecules in the culture filtrate are even smaller, since the sedimentation constants (see Tables I and II) when determined on the filtrates were lower.

Studies on Old Tuberculin and Purified Protein Derivative Fractions Isolated from It.—Attempts were then made to study the smallest potent molecule. The PPD was chosen (15), since it exhibited those properties thought to be evidence of small molecular size. For example, it had been found to pass through membranes of smaller pore size, it was not coagulable by heat, it had a much higher osmotic pressure than the ammonium sulfate fractions, it was non-antigenic with respect to producing the Arthus reaction and gave a very low titer in the precipitin test, while at the same time it was highly potent and a very satisfactory standard diagnostic tuberculin in the skin test (15).

The PPD is a trichloroacetic acid precipitate of old tuberculin (OT) which is prepared by heating in the Arnold sterilizer the entire tubercle bacillus culture, filtering off the dead bacilli through the Seitz filter, adding glycerine and then concentrating the filtrate on the steam bath to one-fifth of the original volume. It seemed advisable, therefore, to study first the original OT, the liquid from which the PPD is isolated. Since large quantities of salts and glycerine were present the solution was thoroughly washed on the ultrafilter before testing in the ultracentrifuge. Much polydispersity was evident, but one main component was found with a mean $s_{20} = 1.37$ (see Table VII). It was apparent that large quantities of very small molecules were also present.

On analysis this washed OT was found to contain 72 per cent

TABLE VII

Comparative Data from Sedimentation, Diffusion, Electrophoresis, and Analyses

| Substance | Per cent concentration used | Buffer concentration | | | s_{20} | D_{20} | Specific volume V | Molecular weights | Per cent carbohydrate | Per cent nucleic acid | Per cent concentration of solution | pH | Ionic strength of solution | Potential gradient | Mobility $\times 10^5$ cm.^{2} sec.$^{-1}$ volt.$^{-1}$ |
		NaCl M	Na$_2$HPO$_4$ M	NaH$_2$PO$_4$ M											
OT	1.0	0.2	0.03	0.02	1.4				72.1	10.0	3.8	8	0.2	5.6	15.9
PPD	1.0	0.2	0.03	0.02	1.1				24.0	21.8	1.0	8	0.02	7.8	49.2 / 36.2 } Two boundaries
PPD-a									12.8	13.0	2.35	8	0.02	9.1	29.3 / 23.1 } Two boundaries
PPD-50d	1.0		0.033	0.002	1.5*	10.2*	0.739	19,000	4.9	3.0					
	1.0	0.2	0.033	0.002	1.8	9.0									
PPD-b2	1.0	0.2	0.033	0.02	1.1	2.1†			9.6	5.7	0.65	8	0.1	8.2	10.1
	0.95	0.2	0.033	0.002	1.5†	2.8									
	0.48	0.2	0.033	0.002											
PPD-b3	0.96		0.0066	0.0004	0.9*	9.3*		16,000	4.4	3.0	1.0	8	0.1	13.8	5.6
	0.69		0.033	0.002	1.1*	9.4*					0.5	8	0.03	8.3	10.7
	1.00	0.2	0.03	0.02	1.3	6.7									
	1.00	0.2	0.033	0.002	1.2	6.2									
	0.20	0.2	0.033	0.002											

	1.0	0.2	0.033	0.002	1.0	10.0	9000	1.2	1.6				
PPD-b3b	1.0												
TPA-30K								2.0	0	1.23	8	0.05	10.6	5.8
Tubercle bacillus nucleic acid								100.0	100.0	1.00	8	0.1	16.8	14.0

* Molecular weights were not calculated from these values because of a possible charge effect.

† Molecular weight was not calculated from these values since its significance would be doubtful on account of inhomogeneity and sedimentation and diffusion anomalies.

polysaccharide and 10 per cent nucleic acid. An attempt was made to separate it into its components by electrophoresis. As can be seen in Table VII, even in the presence of as high a concentration of buffer as $\mu = 0.2$, a boundary migrated to the positive pole with the great velocity of 15.9×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. Because of the concentration and therefore almost black color of the solution it was impossible to determine whether there was more than one additional very heavy and comparatively immobile band.

The analyses of the solution in the various compartments at the end of electrophoresis are given in Table VIII. From these data it is evident that the fast moving component in the top positive compartment was practically all nucleic acid.

TABLE VIII

Analyses of Electrophoretic Fractions of Old Tuberculin

Substance	Per cent total concentration	Per cent of total as nucleic acid	Per cent of total as polysaccharide minus nucleic acid polysaccharide
Original OT solution......................	3.8	10.0	72.1
Top positive compartment.................	0.07	100.0	0.0
Lower positive compartment..............	0.25	76.5	23.5
Bottom U compartment....................	2.67	8.3	68.6
Lower negative compartment..............	2.17	11.5	67.7
Top negative compartment................	1.92	10.2	89.8

PPD was then studied and the sedimentation graph showed it to consist of one main component with $s_{20} = 1.07$, but the curve was asymmetrical. In electrophoresis two components moving with the extremely high velocity of 49.2 and 36.2 cm.^{2} sec.$^{-1}$ volt^{-1} (see Table VII), and one immobile boundary were seen. Considering the foregoing experiment and the apparent concentration of nucleic acid in the PPD, this high mobility may be due to its presence.

Since in previous ultraviolet absorption studies (22) of tuberculin, it had been found that treatment of the PPD with ammonium sulfate removed the substance having the characteristic thymine absorption band, a preparation treated in this way, PPDa, was studied in electrophoresis. The content of nucleic acid was reduced to nearly half

and the mobilities of the boundaries in electrophoresis were correspondingly reduced. These facts, together with the data showing that in general (Table VII) when the content of nucleic acid was low, the mobility was also low, would seem to be good evidence that nucleic acid was responsible for the high mobilities observed. A sample of human type tubercle bacillus nucleic acid[7] was found to have a mobility of 14×10^{-5} cm.[2] sec.[-1] volt[-1] in buffer of one-tenth ionic strength.

Attempts were then made to purify the PPD further by means of ammonium sulfate fractionation. A fraction taken off at one-tenth saturation at pH 4.8, instead of being homogeneous in the centrifuge as was the case with the large molecule, TPA-30b, was found to have a high degree of polydispersity. Finally a fraction, PPD-50d, obtained between 0.2 and 0.5 saturation, proved to be no more homogeneous in the centrifuge than the original PPD, but since the analyses showed that most of the nucleic acid had been removed, it was studied further (see Table VII). It appeared to be nearly homogeneous in diffusion and, therefore, had a probable average molecular weight of about 19,000. Fractionation of the PPD by electrophoresis has not so far proved to be successful in yielding a homogeneous fraction, probably because of the presence of the nucleic acid.

Finally the most promising separation or purification of PPD was made by fractionally precipitating with hydrochloric acid, by use of the hydrogen electrode, as follows. That fraction, precipitating at pH 4.0 and designated as PPD-b2 in Table VII, was removed, precipitated by half-saturation with ammonium sulfate, and then precipitated during electrodialysis. The filtrate from PPD-b2 gave no further precipitate with hydrochloric acid even at pH 3.0, and was therefore precipitated with trichloroacetic acid. It was called PPD-b3 and precipitated when electrodialyzed. During this electrodialysis a fraction, PPD-b3b, passed through the parchment membrane to the cathode. It was recovered by precipitation with trichloroacetic acid.

These three fractions were then studied extensively, as shown in Table VII, since the analyses indicated that a reasonably good purifi-

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[7] Prepared by and given to us by Dr. Robert Coghill.

cation had been accomplished. The sedimentation constants of all of them were very low even in the presence of a sufficient concentration of buffer to suppress all Donnan effect. The sedimentation graphs and results calculated from the diffusion curves indicated considerable homogeneity, in the case of PPD-b3 and PPD-b3b, but not for PPD-b2.

The very low diffusion constant of $D_{20} = 2.1$ for PPD-b2, accompanying such a low sedimentation constant, is noteworthy and strong indication that the molecule behaves abnormally, like thread-like or hydrated molecules. This fraction was difficult to put into solution in buffer at pH 8.0, swelling at first and then showing indication of gelling. However, it showed but one boundary in electrophoresis.

Change in concentration of PPD-b3 altered its diffusion constant very little, whereas change in buffer concentration caused a great difference. At the very low molar concentration of 0.007, a marked charge effect was evident from the fact that the curves tilted to one side (see Lamm, 2[8]). Undoubtedly, of the data obtained, the calculated weight of 16,000 is probably the most nearly correct. This molecule also showed only one component in electrophoresis and an attempt was made to determine its isoelectric point. (See Text-fig. 2, dashed line.) It was not possible to reach the isoelectric point, if there was one, because a precipitate occurred when the solution became more acid than pH 4.4. However, the character of the curve indicates that this molecule bound considerably more base than did the larger molecule, TPA-30K, and that the difference between the two became more rapid around a pH of about 5. The significance of this will be discussed in a future publication, but at present the result may be taken as evidence of the presence of more polar groups in this small molecule than in the larger molecule and fits well with the observation repeatedly made that the conductivity of a concentrated solution of PPD-b3 was greater than that of the buffer in which it was contained.

The PPD-b3b was apparently an homogeneous molecule with a molecular weight of 9000 and the smallest one so far isolated from tuberculin. It is not only an interesting molecule because of its

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[8] Lamm (2), page 99.

homogeneity and small size but also because it has considerably less potency than the other molecules separated from PPD and therefore represents the first stage of breakdown in the molecule associated with loss in potency. This effect is shown in Table VI, referred to earlier. It is also seen in Table IX in another series of experiments, where comparative tests with all the PPD molecules are reported as average reactions observed simultaneously on the same six tuberculous guinea pigs.

PPD-b3 was found to be a poor antigen in the precipitin reaction since 0.009 mg. was required to give a definite minimal precipitate with the same antiserum for which one-tenth this amount was required of the large molecule, TPA-21b.

TABLE IX

Potency of Purified PPD Fractions

Fraction	Average size of skin reactions on six tuberculous guinea pigs	
	24 hrs.	48 hrs.
	mm.	mm.
PPD	16 x 16 x 3.1	15 x 15 x 3.1
PPD-b2	15 x 14 x 3.2	13 x 14 x 3.0
PPD-b3	18 x 17 x 3.0	17 x 16 x 3.5
PPD-b3b	12 x 11 x 2.3	8 x 9 x 1.8

DISCUSSION

It can be seen from these results that there exist in the original human tubercle bacillus culture filtrate molecules with two different sedimentation constants, including a large amount with a constant of 1.9 and a small amount with a constant of 4.9. These may represent molecules respectively of about 15,000 to 18,000 and 70,000 molecular weight. Since an homogeneous substance was isolated with a weight of 32,000, by ammonium sulfate precipitation, it is probable that this molecule is an aggregate of two original ones formed during the isolation. It is significant that this larger molecule exhibits such high antigenic properties, as demonstrated by the striking local anaphylactic type of reaction in tuberculous guinea pigs.

It is possible that an analagous aggregation takes place with the

protein from the timothy bacillus, although in this case the aggregated molecule is only 17,000, and the molecules in the original solution have a still lower sedimentation constant ($s_{20} = 1.4$).

However, the denaturation process, which evidently occurs during this method of purification, as well as the gelling which sometimes appears during simple concentration on the ultrafilter, may be due to continued successive aggregations until particles of many sizes and even of huge dimensions may be formed (as high as $s_{20} = 46$ was observed.)   A similar explanation may be made for the denaturation observed during the crystallization of the tuberculin protein.   At each resolution of the crystals a considerable amount of insoluble material remained on the filter paper.   Furthermore, a batch of crystals which had been recrystallized (23) ten times and which consisted of pure needles and burrs, on standing in the ammonium sulfate medium in the ice box, gradually transformed completely into clearly defined hexagonal plates as shown in the photographs.   It is possible that the needles, because of the close proximity in the compact burrs, coalesced into hexagonal plates, as is suggested by Fig. 2 taken during the period of transformation.   Many of these plates, while having the appearance of beautiful crystals, were insoluble in water and even in phosphate buffer at pH 8.0.

The small molecular size of about 10,000 for the original protein molecules of the bovine bacillus tuberculin may be of significance in connection with two immunological facts previously noted; namely, (a) that the bovine bacillus tuberculin protein is more potent in tuberculous guinea pigs inoculated with human tubercle bacilli than is the human bacillus tuberculin protein (24), and (b) that antiserum to the human bacillus tuberculin protein sometimes gives little or no precipitate with the bovine bacillus antigen, whereas the antiserum to bovine bacillus tuberculin protein usually reacts with the human bacillus antigen (25).

The properties of those molecules considered to be most nearly homogeneous were examined further (see Table X) with the hope of determining their shape and also their degree of homogeneity.   Among other procedures the molar frictional ratios were calculated.   The concentrations of the molecules in solution were also calculated from the sedimentation curves.   Furthermore, the diffusion constants

were calculated from the sedimentation curves, using the time measured from when the centrifuge reached the speed of the experiment. Because of the slow sedimentation only one of the later exposures was used in this calculation. In view of these possible errors, the agreement between calculated and determined diffusion coefficients in all cases except those of the PPD-b2 and PPD-b3 was reasonably close and, therefore, indicated homogeneity. Note in Table X the discrepancy between the actual diffusion constant of PPD-b2 and the apparent one computed from the sedimentation diagram, which indicates heterogeneity.

TABLE X

Homogeneous Molecules Isolated from Tuberculin

Substance	Per cent concentration used	Per cent concentration (calculated from sedimentation diagrams)	D_{20} (found in diffusion)	D_{20} (apparent, calculated from sedimentation diagrams)	s_{20}	f/f_0	Molecular weight
Polysaccharide..............	1.0	0.95	11.0	13.5	1.6	1.5	9000
TPU-Bovine (523).........	0.5	0.42	12.0	11.5	1.6	1.3	10,000
TPA-30b (human).........	0.87		8.2		1.2	1.2	32,000
TTPA-A (timothy).........	1.0	0.46	10.2	13.6	1.8	1.2	17,000
PPD-b3b.................	1.0	0.81	10.0	10.3	1.0	1.6	9000
PPD-b3...................	1.0	0.63	6.7	11.1	1.2	1.9	16,000
PPD-b2...................	0.95	0.48	2.1	14.5	1.5	*	*

* These values were not calculated since their significance would be doubtful on account of inhomogeneity and sedimentation and diffusion anomalies.

Moreover, the f/f_0 ratios were similar to those found for most proteins previously studied, except for the PPD molecules. In the case of the latter, the deviation in shape from the normal compact or unhydrated molecule was marked, especially in the case of PPD-b2. This fits well with the difficulties encountered during work with this fraction, namely, swelling, gelling, and loss in solubility. Probably a distortion of the molecule occurred during the heating in the presence of salts and glycerine and preparation of OT from which the PPD was isolated. Thus an elongation of the molecule was probably produced with exposure of extrapolar groups as is evident from the

base-binding properties of PPD-b3 (see Text-fig. 2, broken line) and a consequent aggregation of these elongated molecules, through the exposed groups to molecules like PPD-b2, as described by Astbury, Dickinson, and Bailey (26) for heated egg albumin.   Dr. O. Snellman of the Physical Chemistry Institute at Upsala kindly made a determination for us on the PPD-b3 preparation by means of the method of double refraction in flow and found a marked effect, indicating very asymmetrical shape.

Since the most probable molecular weight of PPD-b3 is about 16,000, or approximately the same as found for the majority of the molecules in the original medium, and since the original medium has in past researches been shown to be antigenic, it would seem more likely that the lack of antigenicity in PPD-b3 may be due to a distortion or change in configuration of the molecule rather than or as well as to a disaggregation into smaller units.   Moreover, in view of the fact that adsorption to aluminum hydroxide or charcoal renders the non-antigenic molecule antigenic (27) it is probable that some change in physical property rather than the presence of a special constituent is responsible for the antigenic properties.   When the molecule is broken down even as far as 9000, as in the case of PPD-b3b, some loss in tuberculin potency occurs.

SUMMARY

Studies have been made by means of sedimentation in the ultracentrifuge, and by diffusion and electrophoresis, to determine the molecular weights and homogeneity of the tuberculin protein and polysaccharide molecules as found in their natural state in the unchanged filtrates from culture media after growth of tubercle bacilli. These results have been compared with data obtained on fractions isolated by chemical procedures from them or from old tuberculin.

By means of electrophoresis in the Tiselius apparatus it was possible to separate the protein from the polysaccharide, as these two fractions occur naturally in the original culture medium filtrates of acid-fast bacilli.

The protein from the bovine strain of bacillus proved to be homogeneous in sedimentation ($s_{20} = 1.6$), diffusion ($D_{20} = 12.0$) and electrophoresis, with a molecular weight of about 10,000.

The tuberculin polysaccharide isolated in electrophoresis appeared to be practically the same in sedimentation and in precipitin reaction as the polysaccharide isolated by chemical procedure. The latter proved to be homogeneous in sedimentation ($s_{20} = 1.6$) and diffusion ($D_{20} = 11.0$) with a molecular weight of about 9000.

A practically homogeneous protein was isolated from the culture filtrate of the human tubercle bacillus H 37 by fractional ammonium sulfate precipitation, with a molecular weight of 32,000 ($s_{20} = 3.3$; $D_{20} = 8.2$). It was electrochemically homogeneous, with an isoelectric point at pH 4.3 and an isoionic point at pH 4.7. It could be dried or frozen with no loss in homogeneity. It was highly antigenic in the precipitin reaction and produced the anaphylactic type of local skin reaction in tuberculous guinea pigs, in contrast to the true tuberculin type of reaction caused by a purified PPD fraction. Furthermore death resulted in tuberculous guinea pigs from intracutaneous injection of exceptionally small amounts.

A protein with molecular weight of about 17,000 was isolated from the filtrate from cultures of the timothy bacillus.

The nucleic acid originally occurring in old tuberculin (OT) seems to be responsible for the high electrochemical mobility observed. From OT and the PPD made from it, potent but non-antigenic molecules of 16,000 and 9000 weight and with a low content of nucleic acid were isolated. With increase in size these deviated much from the normal compact spherical shape, and aggregation was evident from the tendency toward gel formation. The smallest molecule (9000) was homogeneous ($s_{20} = 1.0$; $D_{20} = 10.0$) and had lost some tuberculin potency.

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EXPLANATION OF PLATE 13

Photographs of tuberculin protein crystals.

FIG. 1. Tuberculin protein crystals when freshly made (needles).

FIG. 2. Intermediate form (spherulite) of tuberculin protein crystals after storage in ice box.

FIG. 3. Hexagonal plates of tuberculin protein crystals after storage for a year.

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2
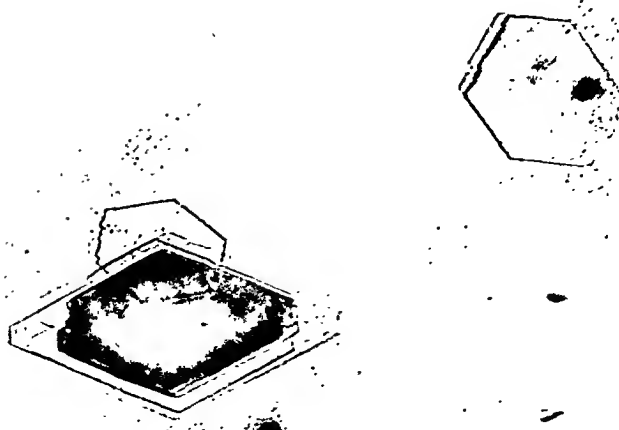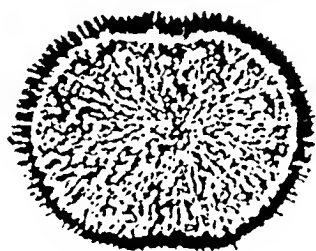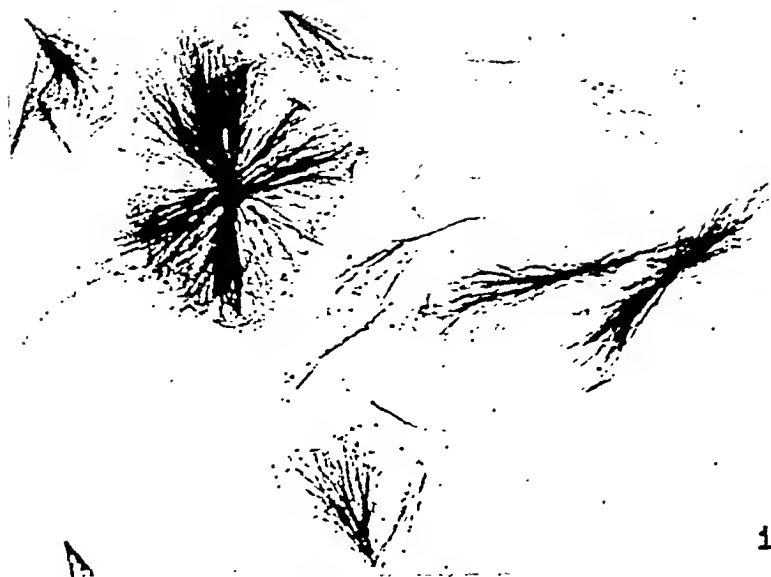
RENAL FUNCTION AS AFFECTED BY EXPERIMENTAL UNILATERAL KIDNEY LESIONS*

I. Nephrosis Due to Sodium Tartrate

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Plate 14

Exact knowledge of the derangements in kidney function due to disease has not been available because of the difficulty in correlating the anatomical lesion in the kidney and the observed changes in kidney function with the current theories of renal physiology. Previous experiments designed to study the functional factors involved in kidney lesions have been complicated, and the results obscured by extraneous effects produced by the nephrotoxic agent. Such experiments have been extensively reviewed by Horn (1) and need not be detailed here.

In 1936, Nicholson, Urquhart and Murray reported to the Toronto Biochemical Society, a method for the perfusion of one kidney of an animal without allowing the perfusion fluid to enter the general circulation. The circulation of the kidney was then restored and the kidney replaced in the animal. At the end of 48 to 56 hours this procedure resulted in a degenerative lesion of the proximal convoluted tubules (a nephrosis) with albumin and casts in the urine. The uninjected kidney presented no pathological changes.

This method permits one to observe the functional changes due to the kidney damage alone, eliminating the possibility of changes in function due to the production of more generalized lesions. It also provides a most accurate control, namely a normal kidney in the same animal subjected to identical experimental conditions.

Method

Operative Technique.—Moderately thin medium sized female dogs were chosen. Under ether anaesthesia the left kidney was exposed through the flank and the vessels freed from the surrounding structures. The artery and vein were separated and the artery stripped just sufficiently to permit of easy injection. Care was taken to avoid unnecessary injury to nerves.

Bulldog clamps were placed on the artery and vein. 200 to 250 ml. of an aqueous solution of 7.5 per cent anhydrous sodium tartrate, 1 per cent sodium
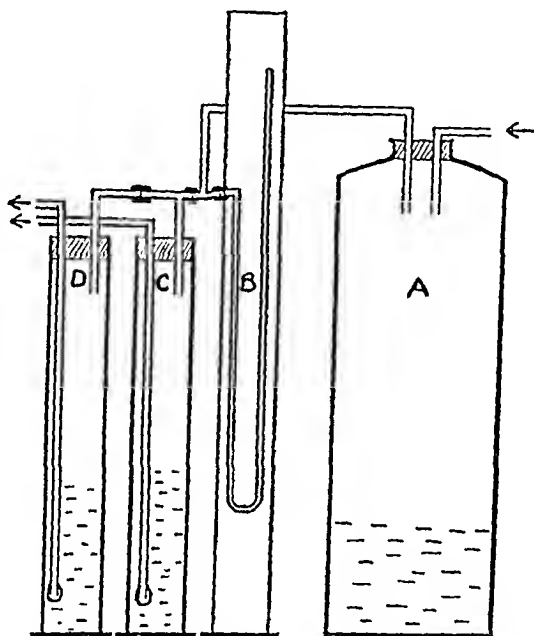
TEXT-FIG. 1. Apparatus devised for injection of kidney under constant pressure.

A = pressure bottle.
B = mercury manometer.
C = graduate containing sodium tartrate.
D = graduate containing citrate saline.

citrate solution and 0.9 per cent saline were injected into the renal artery through a No. 19 or No. 20 needle. A constant pressure of 200 mm. of mercury and a rate of 35 to 40 ml. per minute were maintained (Text-fig. 1). This was followed by 50 ml. of a 1 per cent sodium citrate solution in 0.9 per cent saline. The injection fluids were allowed to escape through a puncture in the renal vein and were taken up by suction, none being allowed to enter the peritoneal cavity. The circulation to the kidney was then restored and as soon as bleeding became controlled the kidney was replaced and the incision closed in the usual manner.

In the majority of experiments after an interval of 48 hours the bladder was

opened under light nembutal anaesthesia and the urine from each kidney collected through ureteral catheters. Observations were made on three control groups:

1. Variations between the two kidneys were determined under nembutal anaesthesia in normal animals.

2. In a small group to assess the effect of anaesthesia, the bladder was exposed, divided and sutured in such a way as to provide separate pouches for each ureter. These were drained by means of Pizer catheters connected to a specially devised constant suction apparatus, arranged so that the animal had considerable freedom of movement (Text-fig. 2). This made possible the collection of the urine from
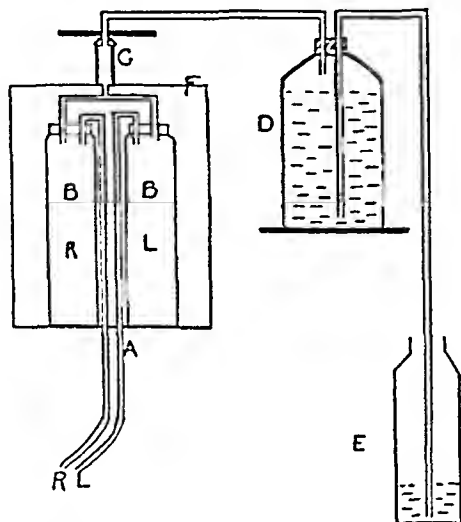
TEXT-FIG. 2. Apparatus devised for collection of urine from separate bladder pouches over long periods of time.

A = tubes leading from bladder pouches.
B = collecting bottles.
C = swivel mechanism.
D and E = syphon system.
F = carriage to hold collecting bottles.

each kidney over prolonged periods. Individual clearances could be obtained before injection and at varying intervals following the injection without the use of anaesthesia.

3. The effect of possible nerve damage was observed by (a) carrying out the preliminary procedure on the left kidney alone without subsequent injection and (b) carrying out the same procedure on the right kidney immediately after the left was injected. In addition in a few animals the effect of a fairly complete denervation of one kidney was observed.

Clearance Technique.—Clearances were determined 48 hours following the injection. The clearance of urea, xylose, creatinine, inulin and phenol red were studied. Urea and xylose clearances were done simultaneously with creatinine and/or inulin. Since in the dog creatinine and inulin clearances are identical (2), the inulin clearance was done simultaneously with that of phenol red to avoid the necessity for using a compensating colorimeter.

The substances to be studied were given with an initial intravenous injection of 300 ml. of 5 per cent glucose in order to insure a flow of urine well above the augmentation limit. The amounts used varied to some extent with the size of the animal as follows: Creatinine 2 to 3 gm., xylose 5 to 7.5 gm., inulin 2 to 3 gm., phenol red 50 to 75 mg. Following the first 30 minute period a continuous intravenous injection of normal saline was given containing additional amounts of these substances. In 500 ml. of 0.9 per cent saline it was necessary to have xylose 5 gm., inulin 1 gm. and phenol red 125 mg., to keep the blood level of these substances fairly constant, according to the recommendation of Smith (3). During the continuous administration of normal saline two or more clearance periods of 30 minutes were run. Following collection the urine was analyzed for its content of the substances cleared. In all cases plasma was used for the estimation of blood level of these substances, the blood being drawn and centrifuged under oil. Blood samples were taken at the beginning, midpoint and end of the first period and at the beginning and end of each subsequent period.

Analytical Technique.—Urea was determined by the method of Van Slyke and Cullen (4). Sugars were determined on 1 ml. portions by the Harding and Downs modification of Shaffer-Somogyi reagent (5). Creatinine was determined by the method of Folin and Wu (6), using the Evelyn photoelectric colorimeter (7). Phenol red was determined by the addition of 5 ml. of 25 per cent NaOH to 10 ml. of the unknown solution and measuring the color developed in the Evelyn colorimeter using the No. 540 filter.

Plasma urea was estimated directly using 3 ml. of plasma. Folin-Wu filtrates were used in the determination of xylose, inulin, creatinine, and phenol red. Xylose was estimated as the residual reduction after yeast fermentation. Inulin was estimated as the increased reduction due to hydrolysis with N/1 H_2SO_4.

Plasma chloride was estimated by means of a modification of the Whitehorn-Volhard (8) titration as follows: 20 ml. of plasma filtrate were taken and 20 ml. of silver nitrate reagent were added to it. After standing the mixture was filtered. 20 ml. of this filtrate was titrated with NH_4CNS as described in the original method.

Urine.—Urea was estimated as for plasma using 1/100 dilutions of the urine.

Xylose was estimated as above using 1/100 to 1/400 dilutions according to the concentration of the sugar. In dilutions of 1/200 or greater fermentation was unnecessary as normal urine reducing substances were diluted out beyond the sensitivity of the reagent.

Inulin was determined as in plasma using 1/400 or 1/800 dilutions.

Phenol red was estimated as in plasma using 1/400 dilution.   Higher dilutions were employed when necessary.

Urine chloride was estimated by a modification of the Volhard-Harvey (9) titration, 5 ml. of urine were pipetted into a 100 ml. volumetric flask; 5 ml. of HNO_3 conc. were added and heated in a boiling water bath.   By this procedure phenol red was destroyed.   After cooling 10 ml. of the reagent were added.   After standing the precipitate was removed by filtration.   25 ml. portions of the clear filtrate were titrated with NH_4CNS as described in the original method.

Histological Technique.—Kidneys were removed from the anaesthetized animal, cut into thin slices, examined for gross changes, and fixed in neutral formalin. In a number of instances pieces were fixed in Zenker's solution without acetic acid and in Bensley's A.O.B. fluid.   Representative portions were embedded in paraffin by the usual methods and sections of from 5 to 10μ cut.   The formalin-fixed sections were stained with haematoxylin-eosin, the Zenker-fixed sections with Heidenhain's azan-carmin modification of Mallory's aniline blue stain according to McGregor (10) and those fixed in A.O.B. fluid were stained with iron haematoxylin.   Sections of the kidneys of a number of rabbits which had been injected with sodium ferrocyanide as described by Gersh and Stieglitz (11) were prepared by Gersh's (12) modification of the Altmann technique for fixation by drying while freezing.   The ferrocyanide was stained by the Prussian blue reaction according to the directions of Gersh and Stieglitz (11).   A few minor modifications were introduced.   The sections were dropped into chilled isopentane as recommended by Hoerr (13).   The isopentane was chilled in liquid air in an apparatus arranged so that there was no possibility of the pentane and liquid air mixing.   The frozen sections were then kept at a temperature of $-30°C$. by immersing the vessel in which they were to be dehydrated in a mixture of alcohol and water with a freezing point of $-35°$ to $-40°$ chilled by being packed in solid CO_2 and kept from freezing by constant agitation by a stream of compressed air. Depending on the volume of the stream any desired temperature between $-25°$ and $-35°$ could be maintained.   5 pounds of solid CO_2 would last for 10 to 12 hours if the container was well insulated with felt.[1]

RESULTS

Injection of 7.5 per cent sodium tartrate in the manner described produced a degenerative change in the cells of the proximal convoluted tubules characterized by swelling, blurring of the outlines, marked

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granulation with intensification of the acidophilic staining, destruction of portions of the brush border and desquamation of cellular material into the lumens of the tubules. The damage did not affect the other portions of the tubules to any appreciable extent. Casts were found in many of the tubules. The glomeruli appeared normal, careful examination showing no differences between those in the damaged and those in the control kidneys. In the urine from the damaged kidney, albumin and cellular, granular and hyaline casts were found, the cellular casts being tubule cell casts.

This damage affected the excretion of water, chloride, nitrogenous substances, inert sugars and ferrocyanide. The excretion of these

TABLE I

Variations in Water and Chloride Excretion under Conditions of No Diuresis

Experiment No.	Urine volume		Decrease from damaged kidney (left)	Excretion of chloride	
	R	L		R	L
	ml. per 30 min.	*ml. per 30 min.*	*per cent*	*mg. per 100 ml.*	*mg. per 100 ml.*
7	10	6.3	37	112	187
10	6.4	5.9	8	732	912
29	13.0	7.5	42	60	132
34	13.5	10.5	22	76	100
40	4	1.5	63	10	600
86	11.5	8	30	116	176
96	6.5	2.4	63	—	160
97	5.3	3.9	26	—	—

substances was further altered by the experimental conditions under which the damaged kidney functioned, *i.e.*, no diuresis, sugar diuresis or salt diuresis.

When no diuresis was present the fluid output from the damaged kidney was always considerably less than from the normal. Table I shows the average relative output of urine found in a series of animals, the volume of urine from the damaged kidney (left) being reduced from 8 to 63 per cent. The concentration of chloride under these conditions was always increased from the damaged kidney and in most instances the absolute amount excreted was also greater than the normal (Table I).

When a diuresis was induced by the intravenous injection of 5 per

cent glucose, the fluid output from the damaged kidney increased to an amount approaching the normal even though the amount from the normal kidney was also increased (Table II). Under these conditions also the amount of chloride excreted by the damaged kidney exceeded that put out by the normal (Table II).

When a diuresis was induced by a continuous intravenous injection

TABLE II

Variations in Water and Chloride Excretion with Glucose Diuresis

Experiment No.	Urine volume		Decrease from damaged kidney (left)	Excretion of chloride	
	R	L		R	L
	ml. per 30 min.	*ml. per 30 min.*	*per cent*	*mg. per 100 ml.*	*mg. per 100 ml.*
4	90	54.0	51	280	300
10	10.5	7.5	29	1068	1028
14	20	6.9	65	175	265
20	15	23.0	53*	666	1004
21	·17	22.0	30*	86	239
22	26	23.5	10	40	196
23	21	10.5	50	—	120
37	14	17.5	25*	60	202
49	20	11.0	45	68	136
81	30	24.5	18	64	76
85	15	15.0	—	52	44
86	11.5	8.0	30	116	176
87	10	16.0	60*	72	16
90	19.5	15.5	20	56	20
91	9.7	8.3	15	40	40
92	6.5	11.5	77*	56	440
95	8.2	18.0	120*	120	344
98	14.9	17.7	18*	32	192
99	17.4	17.7	2*	60	64

* Increase.

of 0.9 per cent sodium chloride the fluid output from the damaged kidney was at first always considerably greater in amount than from the normal. If the intravenous injection was continued for a sufficient length of time the urine output from the normal kidney approached or passed that from the damaged kidney (Table III). The output of chloride from the damaged kidney was greater than from the normal kidney. As the intravenous sodium chloride injection progressed

TABLE III

Variations in Water and Chloride Excretion with Normal Saline Diuresis

Experiment No.	Urine volume		Change from damaged kidney (left)	Excretion of chloride	
	R	L		R	L
	ml. per 30 min.	*ml. per 30 min.*	*per cent*	*mg. per 100 ml.*	*mg. per 100 ml.*
29	8	17	112*	476	556
	11	30	173*	1284	632
20	23	60	200*	764	884
21	18	30	67*	94	327
22	32	30	7	56	88
	12	9.5	20	108	228
23	34	24	29	294	98
34	12.5	27.5	120*	108	256
	51	91	98*	80	140
37	17	75	340*	360	744
	14.5	57	225*	704	824
	14.5	35	140*	1184	1168
38	18	27	50*	1192	996
	31	47.5	53	844	672
40	19	86.5	360*	536	456
49	34	30.5	10	1648	1066
81	9.5	15	58*	676	520
	11	17	55*	732	580
85	18	20	11*	208	376
	43	52	21*	340	472
86	12	22	83*	912	660
	5.5	7.5	37*	288	200
90	20	16	20	556	1112
	21.5	15	25	1004	608
	25.8	26.3	2*	736	500

* Increase.

TABLE III—*Concluded*

Experiment No.	Urine volume		Change from damaged kidney (left)	Excretion of chloride	
	R	L		R	L
	ml. per 30 min.	*ml. per 30 min.*	*per cent*	*mg. per 100 ml.*	*mg. per 100 ml.*
91	20.3	20.9	3*	640	400
	41.5	53.7	30*	240	400
92	9.9	12.7	28*	180	304
	13.2	9.7	26	312	416
96	8.7	9.1	5*	368	272
	12.9	9	22	1136	768
98	9.9	29	200*	408	688
	15.9	50.3	218*	668	400
99	8.2	15.8	92*	100	100
	11.6	31.6	170*	80	188

there was at first an increase in the amount of chloride excreted by both kidneys with the amount from the normal kidney eventually exceeding that from the damaged kidney (Table III).

That this effect of intravenous sodium chloride producing results comparable with those found in the denervated kidney (Marshall and Kolls) (14)), was not due to injury to renal nerves during the operative procedure, is shown as follows:

1. The urine from the damaged kidney was always less in amount when there was no diuretic stimulus (Table I), whereas the denervated kidney excretes larger amounts of urine under all conditions (Marshall and Kolls (14)), (Table IV).

2. In certain control experiments the preliminary operative procedure was carried out on the left kidney, no injection being given. There was no difference in the function of the two kidneys (Text-fig. 3).

3. In certain other control experiments the preliminary operative procedure was carried out on both kidneys, the left kidney only being injected. The effect on water and chloride excretion (as of the other substances tested) was not different from the effect on those substances as shown above (Text-fig. 3).

4. It is significant that Underhill, Wells and Goldschmidt, quoted

TABLE IV

Effect of Denervation on the Excretion of Water and Chloride

Experi-ment No.	Procedure	Urine volume		Increase from denervated kidney		Excretion of chloride	
		R	L	R	L	R	L
		ml. per 30 min.	*ml. per 30 min.*	*per cent*	*per cent*	*mg. per 100 ml.*	*mg. per 100 ml.*
82	Left denervated	20.5	32.5		60	48	212
		16*	43.5		170	112	704
		22	102		365	320	800
83	Right denervated	31	19.5	61		164	28
		15*	6	150		1220	460
		31	12	160		1368	1064
		57	17	240		1328	1544
84	Left denervated	20	28.5		42	116	316
		9*	32		255	752	968
		7	23		230	880	1020
		14	59		320	876	960

* Intravenous saline started.
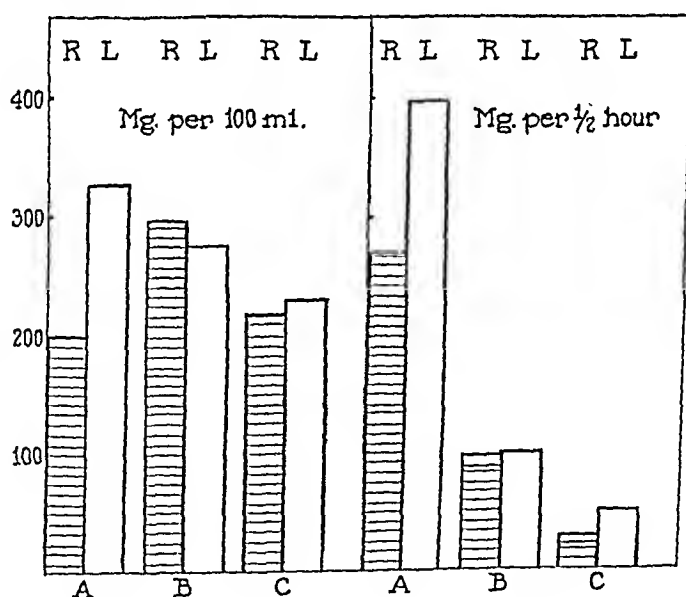
TEXT-FIG. 3. The effect of the sodium tartrate injection on the excretion of chloride.

A = right renal artery stripped; left kidney injected.

B = left artery stripped but not injected.

C = no stripping or injection.
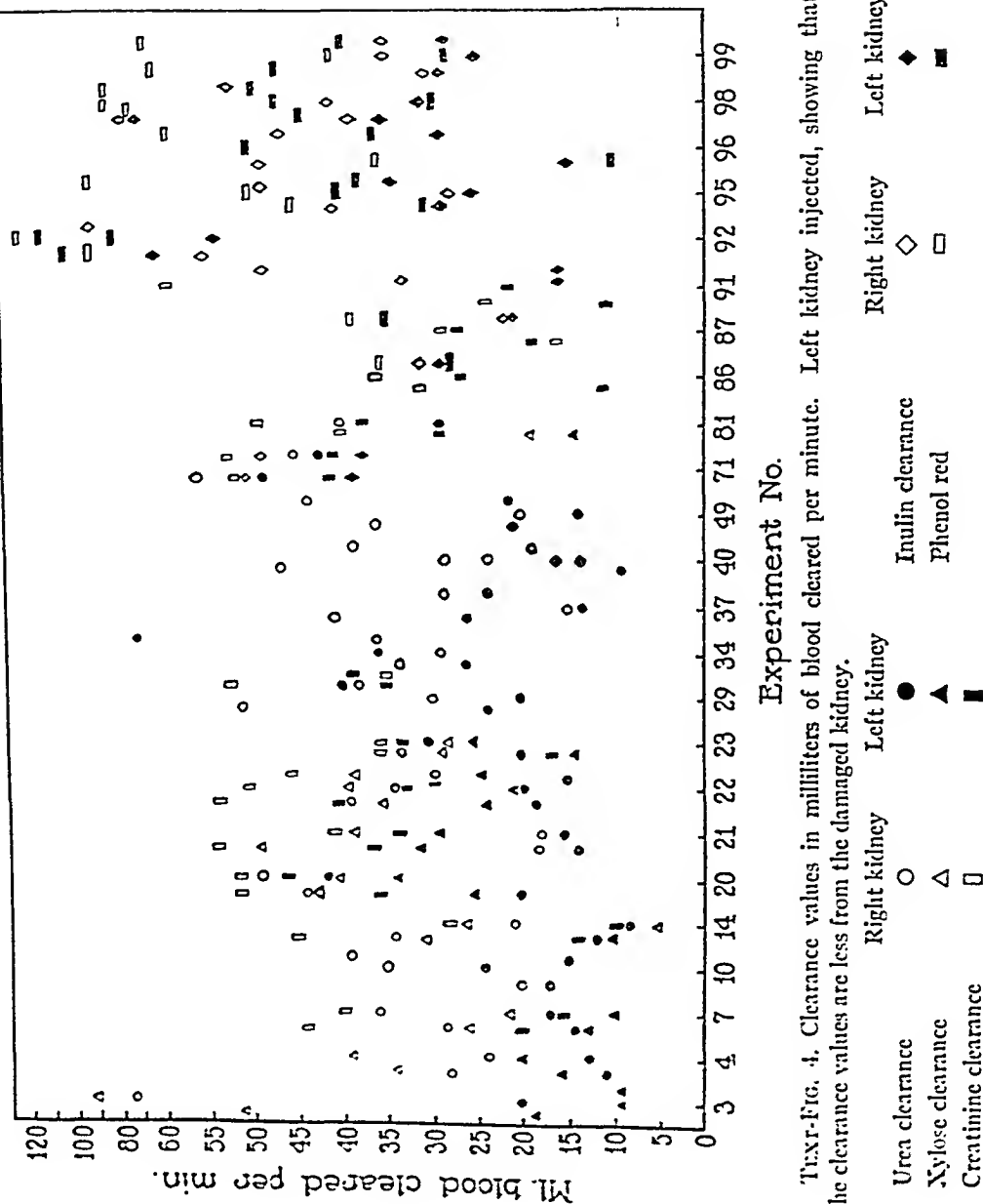
Text-Fig. 4. Clearance values in milliliters of blood cleared per minute. Left kidney injected, showing that the clearance values are less from the damaged kidney.

by Horn (1), found that the subcutaneous injection of sodium tartrate in rabbits produced necrosis of the convoluted tubules with an increased chloride excretion.
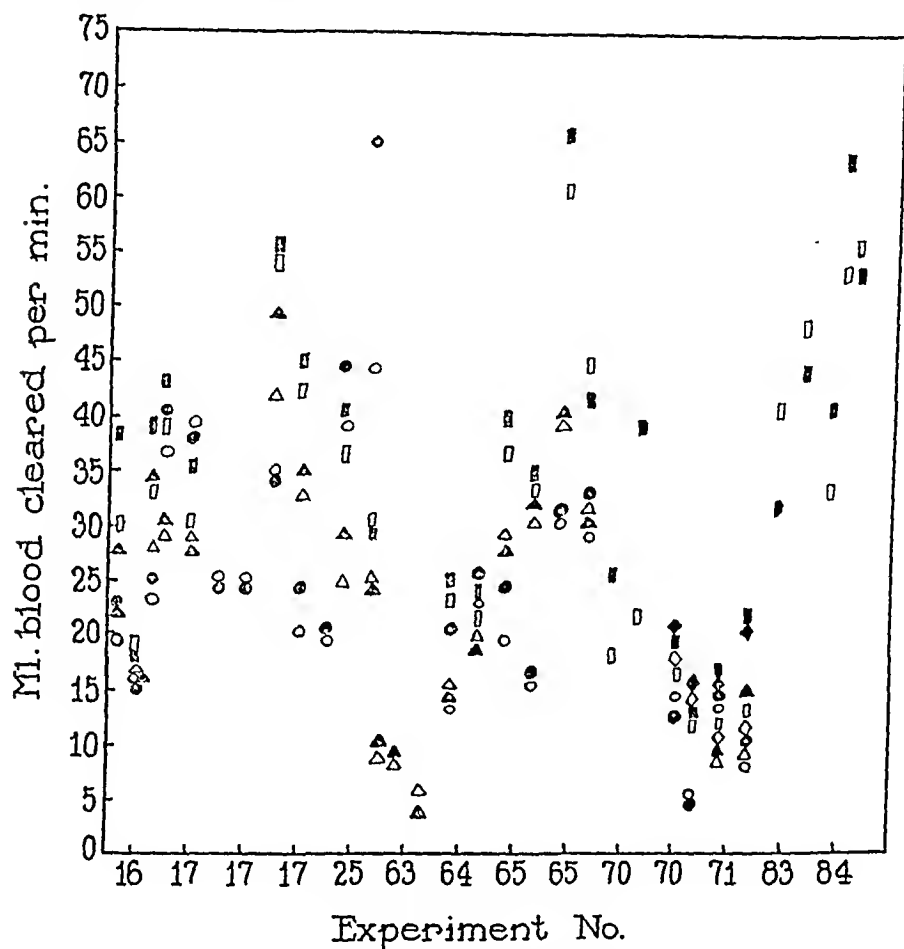
TEXT-FIG. 5. Clearance values in milliliters of blood cleared per minute; both kidneys normal, showing the normal physiological variation.

	Right kidney	Left kidney
Urea clearance	○	●
Xylose clearance	△	▲
Creatinine clearance	◻	◼
Inulin clearance	◇	◆
Phenol red	▭	◼

The ability of the nephrotic kidney to excrete nitrogenous substances (urea and creatinine) and inert sugars (xylose and inulin)

is decreased (Text-figs. 4 and 5). Clearances of these substances calculated for the individual kidney are for the most part smaller from the damaged kidney. In some few instances the clearances of creatinine and inulin, for a single 30 minute period, may be the same. This does not hold true for urea. On the whole the diminution of
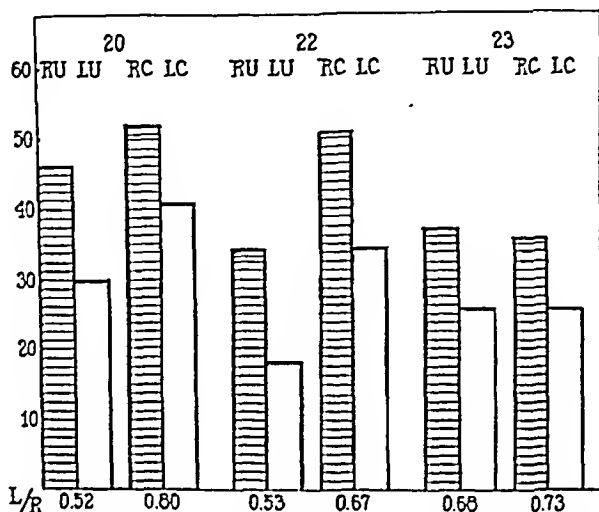
TEXT-FIG. 6. The decreased excretion of urea as compared to creatinine in three typical experiments.

RU = right urea clearance.
LU = left urea clearance.
RC = right creatinine clearance.
LC = left creatinine clearance.
$L/R = \dfrac{\text{Left clearance}}{\text{Right clearance}}$.

the urea clearance is greater than the decrease in the creatinine clearance as shown by the ratio of

$$\frac{\text{Cm. urea–nephrotic kidney}}{\text{Cm. urea–normal kidney}} \quad \text{and} \quad \frac{\text{Cm. creatinine–nephrotic kidney}}{\text{Cm. creatinine–normal kidney}}$$

(Text-fig. 6). This ratio in the case of urea, however, is markedly elevated by diuresis in the damaged kidney.

The phenol red clearances are strikingly affected in the damaged kidney. The marked decrease in phenol red clearance was much

greater in degree than the decrease in inulin or creatinine clearance. A comparison of the phenol red to inulin ratios is shown for the two kidneys (Table V). This ratio is much lower for the damaged kidney than for the normal.

The limited number of ferrocyanide clearances done gave results similar to the inulin and creatinine clearances. The elimination of ferrocyanide was more extensively studied in sections obtained by the freezing-drying technique. In such sections of normal kidney, ferrocyanide was found in the glomerular tufts and spaces and in the

TABLE V

Relationship between the Excretion of Phenol Red and Inulin from the Damaged Kidney

Experiment No.	Clearances				Ratio phenol red to inulin	
	Phenol red		Inulin			
	R	L	R	L	R	L
95	44	30	42	29	1.05	1.03
	50	40	26	27	1.95	1.50
	100	36	48	34	2.04	1.07
98	65	44	39	36	1.67	1.22
	83	30	42	30	1.98	1.00
	83	48	53	47	1.57	1.02
99	60	47	31	29	1.93	1.55
	41	28	35	26	1.17	1.08
	62	39	35	28	1.75	1.39

lumen of all portions of the tubule, the picture being essentially the same as that described by Gersh and Stieglitz (11). Sections from the damaged kidney were strikingly different. The appearance of the glomeruli was unchanged but the convoluted tubules showed a certain amount of raggedness and loss of continuity of the brush borders with deposits of ferrocyanide throughout the whole thickness of the tubular wall. The ferrocyanide when revealed by the Prussian blue reaction was seen in the cells as a diffuse bluish staining and as granular deposits. In the loops of Henle, distal convoluted tubules and collecting tubules the ferrocyanide was confined to the lumen as in the

normal kidney (Figs. 1 and 2). From this it is evident that the damage is limited to the proximal convoluted tubules; that it is of such a nature that it renders these cells permeable to ferrocyanide, allowing this substance to diffuse back through the walls. It is probable that this back diffusion accounts for the decreased clearance of ferrocyanide.

Determinations of the ammonia in the blood from the renal veins, arteries and in the urines from each kidney were done on a limited number of animals. No differences were detected between the damaged and the undamaged kidney.[2]

DISCUSSION

The changes in function herein noted are due to the damage produced in the kidney. The method of injection precludes injury to other structures. The presence in the animal of a normal kidney prevents the retention of metabolites which might influence kidney function. In addition it is to be noted that the changes found are greater than the physiological variations when both kidneys are normal (Text-figs. 3 and 5).

From the histological standpoint the glomeruli were unaffected, the lesion being confined to the proximal convoluted tubule. A comparison of the various clearances shows that the damaged kidney usually has a lower clearance rate for each substance than the normal. This reduction in the case of creatinine and inulin (which in the dog are excreted entirely by the glomerulus) was the same. The clearance of phenol red, 90 per cent of which is secreted by the tubules (Marshall et al. (15, 16) and Shannon (17)) and later shown to be limited to the proximal convoluted tubules (Gersh, 1934 (18)), is reduced to a much greater degree (Table V). The damage to the proximal convoluted tubules therefore has definitely impaired their function.

It has been demonstrated by Van Slyke et al. (2) that the clearance of creatinine and inulin in the dog represents the glomerular filtrate. The decrease in the clearance of these substances from the damaged kidney might be taken to indicate glomerular damage of such a nature that it could not be detected by the histological methods at present

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[2] Unpublished results of work done in this laboratory by Mr. A. G. Gornall.

available.   Such an assumption is unnecessary as is shown by the histological studies on the excretion of sodium ferrocyanide.   These experiments demonstrate clearly that the impermeability of the cells of the proximal convoluted tubule to ferrocyanide is lost to such a degree that a marked back diffusion of the salt takes place.   It is altogether likely that there is a similar alteration in permeability to creatinine and inulin.   This would adequately explain the decrease in the clearances of these substances from the damaged kidney.   The clearance of inulin or creatinine in these cases therefore does not represent the glomerular filtrate.

The slightly greater decrease in urea excretion, which occurred under some conditions, either may be due to increased back diffusion of urea or may indicate a possible secretory action of the proximal tubule, impaired under these circumstances.

The decreased water excretion under "resting" conditions may also be explained by increased back diffusion.

The relative inability of the nephrotic kidney to hold back chloride points to the possibility that the proximal tubule plays an active part in reabsorbing chloride and probably sodium.   The increased output of urine during salt diuresis is probably explained by the fact that the sodium and chloride not reabsorbed hold with them varying amounts of water.   This causes a greater diuresis from the damaged kidney than occurs from the normal.

The results of the ammonia determinations indicate that the proximal convoluted tubule is probably not the site of ammonia formation.

SUMMARY

1. A method is described for the production of a nephrosis in one kidney of an experimental animal.   The normal kidney in the same animal is available for a control.

2. The nephrosis produced by the injection of 7.5 per cent sodium tartrate is limited to the proximal convoluted tubule.   There is no histological evidence of glomerular damage.

3. This damage results in a disturbance of water and chloride excretion as well as a decrease in the clearances of urea, xylose, inulin, creatinine and phenol red, and is observed under conditions of no diuresis, sugar diuresis and salt diuresis.

4. Excreted ferrocyanide is found in the cells of the proximal tubule of the damaged kidney.   It is not present in these cells in the normal kidney.   This is taken to be evidence of back diffusion of ferrocyanide through the damaged cells.

5. The decrease in clearance of creatinine and inulin, which in the dog represent glomerular filtrate, may therefore be explained by back diffusion and is not necessarily due to glomerular change.

6. The phenol red clearance from the damaged kidney is diminished in relation to inulin. This is further evidence of depressed tubular activity.

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EXPLANATION OF PLATE 14

The excretion of ferrocyanide.

FIG. 1. Damaged kidney (left). (*a*) Glomerulus. (*b*) Proximal tubule, showing the presence of ferrocyanide within the cells. (*c*) Distal tubule.

FIG. 2. Normal kidney (right). (*a*) Glomerulus. (*b*) Proximal tubule. (*c*) Distal tubule.

1a

1b

1c

2a

2b

2c

E Blackslick
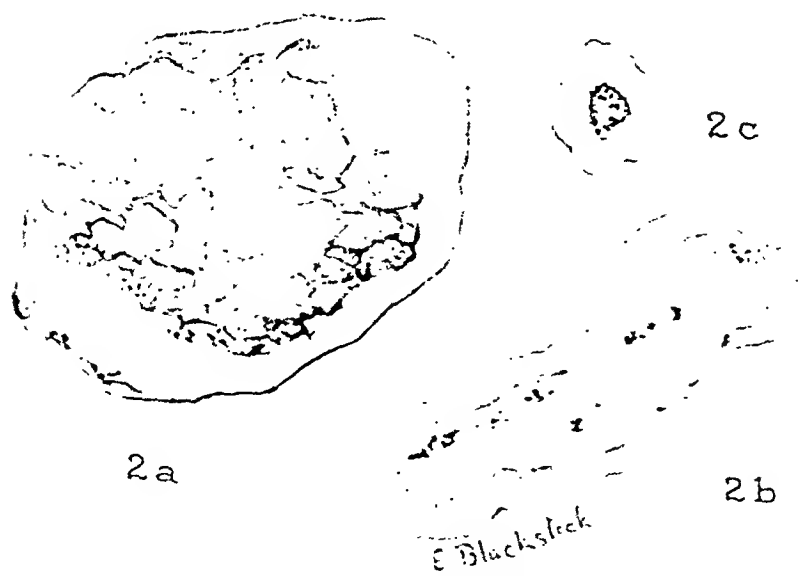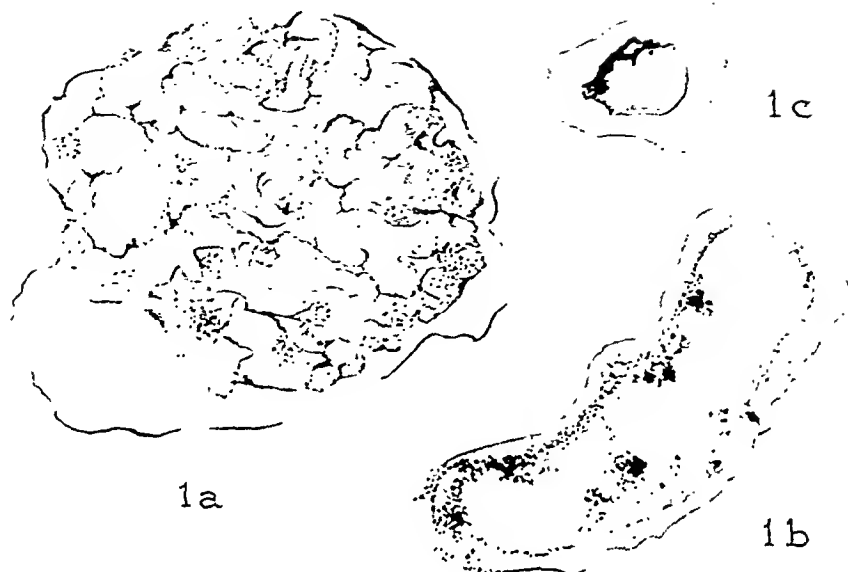
CARCINOMA IN THE LEOPARD FROG: ITS PROBABLE CAUSATION BY A VIRUS[*]

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PLATE 15

For the experimental study of neoplastic diseases, warm blooded animals such as rodents and fowls have generally been used. The more primitive cold blooded vertebrates have been neglected because of the belief that among them tumors are rare and consequently not readily available for investigation. Recently, however, it has been shown that the leopard frog (*Rana pipiens*) is commonly affected with a carcinoma of the kidney. This is a particularly interesting tumor as its cell nuclei frequently contain large acidophilic inclusions such as suggest the presence of a virus (1). In the present paper an account is given of transmission experiments the results of which make it probable that this carcinoma is, in fact, induced by a virus.

The general characteristics of the spontaneous tumors, of which somewhat over 600 have been examined in this laboratory during the past 5 years, are briefly as follows:

The growths occur in one or both kidneys as solitary or multiple, white, solid or partially cystic growths varying in size from small nodules to large irregular masses several times the size of the kidney which they replace. The larger and presumably more rapidly growing tumors not uncommonly metastasize.[1] Histo-

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[1] This fact has developed from more recent studies and is contrary to our earlier report based on the finding of but three examples of metastasis among 276 cases of these tumors (2). But in a subsequent group of 146 tumors there were 17 with metastasis. The difference between the two groups lies in the much greater proportion of large tumors in the second group. A discussion of the incidence of metastasis of these tumors will be given elsewhere (3).

logically, the majority of the tumors have the appearance of adenocarcinoma. The component epithelial cells are quite atypical and much larger and more basophilic than normal kidney cells; usually they are crowded in disorderly multiple layers around irregularly shaped gland-like acini. Numerous mitotic figures denote active proliferation; the stroma is scanty and poorly vascular; a capsule is lacking; and marginal extensions of the tumor infiltrate and destroy the adjacent kidney.

In a smaller group the component cells are less atypical, the tumor tubules are single layered and more orderly, there are few mitotic figures, and, while no capsule is present, no extension of the periphery occurs. A frequent variation from this adenomatous growth is cystic dilatations with papillary projections into the cyst.

All gradations are found between the frankly malignant, invasive, and destructive adenocarcinoma and the structurally benign adenoma, cystadenoma, and papillary cystadenoma. In general the larger tumors nearly always have a malignant appearance, though many minute nodules are also evidently carcinomatous. The neoplastic disease, once established, appears to be progressive, for tumors with evidence of recession such as atrophy, extensive necrosis, and marked overgrowth of stroma are uncommon.

The outstanding characteristic of the frog tumor is the frequent presence of acidophilic intranuclear inclusion bodies which in their general appearance are like those found in herpes and certain other diseases known to be due to viruses. They invariably are confined to the cells of the tumor and have never been observed in normal renal epithelium of tumor-bearing kidneys, nor in the cells of other organs. In their typical form they are conspicuous and readily recognizable, and in such form they are observed within most of the tumors. It is obvious that there must be developmental stages, and the appearance of the early stages is still a matter of doubt. Moreover there appear to be seasonal variations; at least the inclusions are more frequent in winter and spring than in summer and autumn. Their common association with tumor cells, and their constant absence from related normal epithelium make it unlikely that they represent a virus which has only secondarily invaded an established neoplasm.

Material and Methods

Three series of transmission experiments are reported in this paper. In the first series the inoculation consisted of living tumor; in the second of desiccated or glycerinated tumor. The frogs used were adult leopard frogs (*Rana pipiens*) of both sexes and of average size, which came from New England. In a third series, alien species were used.

The frogs were kept in groups not exceeding 20, in large glass tanks. The tanks contained smooth rocks, and water of sufficient depth for frogs to submerge

themselves completely; change of water was provided by a constant inflow and outflow. The colony was housed in a cool basement room designed as an amphibian vivarium, in which the temperature ranged around 45–50°F. in the winter, and around 65–70° in the warmer months. Every group was inspected twice daily, and great care was taken to remove dead animals promptly. The frogs were fed, usually twice a week, with earthworms, mealworms, or insects; this food they learned to take readily. When living food was not available, bits of liver were fed by hand. During the coldest months no food was given. It proved possible to maintain the animals for from 6 months to more than a year in fairly good condition, excepting that despite isolation in small groups, recurrent epidemics (usually of red leg) destroyed considerable numbers.

The methods employed for inoculating living tumor were (a) to introduce small fragments, cut from healthy appearing areas with sharp scissors, by means of a hollow needle provided with a well fitting plunger (Bashford needle); or (b) to inject with a syringe a suspension of cells prepared by squeezing the tumor through a finely meshed sieve, or by grinding it in a rough bottomed mortar. Enough amphibian Ringer's solution was added to the cell mash to bring each dose of the suspension to 0.5 cc.

Desiccated tumor was prepared by the Flosdorf-Mudd lyophile process (4). The minced material was frozen at approximately −80°C. in a mixture of cellusolve and solid CO_2, and dried by high vacuum distillation from the frozen state; the containers were then sealed under vacuum and stored at refrigerator temperature for 2 or 3 weeks. For use the dried material was reduced to a fine powder by grinding and was suspended in sterile water; 0.5 cc. of the suspension was injected.

One group of frogs received an emulsion of a glycerinated tumor. The material, which has been stored for 20 days in 50 per cent glycerin at refrigerator temperature was washed repeatedly in amphibian Ringer's solution, and then ground to a fine emulsion of which 0.5 cc. was injected.

In the preparation of the tumors by these several methods aseptic precautions were observed. However, since it is virtually impossible to sterilize the skin of frogs without injuring it severely, the site of inoculation was merely moistened with 70 per cent alcohol both before and after inoculation.

The tumors used in the experiments varied greatly in size; many were small, early growths and furnished only sufficient material for inoculating relatively few animals, about 10 to 15; other tumors were large and represented an advanced stage of the neoplastic process. Now, since it seemed possible that in their various stages of growth, the tumors might vary in transmissibility, it became necessary to make a choice between using a variety of tumors with which to inoculate relatively small groups of frogs, or to rely on a few large tumors with which many frogs could be inoculated. Because frogs of uniform age and of pure breed were not easily available it seemed best, in these experiments at least, to choose the first of these procedures, and by using a variety of tumors, minimize the effect of variable factors in both tumors and animals. Thus, a total of 810 frogs, in groups of

from 10 to 40, approximately, received inoculations from 44 different tumors. (In this total are not included groups which because of infection failed to survive 6 months following inoculation.) As controls, 953 frogs were maintained under precisely the same conditions as prevailed in the experimental series.

For histological study, material was fixed either in formalin or in Susa fluid, and stained usually with hematoxylin and phloxin, or with a modified Giemsa solution.

Results with Living Tumor

Solid fragments or cell suspensions were inoculated into the dorsal or ventral lymph sacs (*i.e.*, subcutaneously), in the abdominal cavity, in the muscles of the thighs, or intracranially (by injection through an orbital plate). At none of these several sites did a progressive tumor develop. Usually, the injected material was rapidly destroyed and resorbed. Occasionally, fragments were found at autopsy for as long as 4 months, particularly those introduced in the lymph sacs. Such grafts had become attached and were vascularized. Some had undergone slight increase in size, but, histologically, all gave evidence of regression rather than of proliferation. While there were isolated well preserved areas of carcinomatous appearance, in which some of the cells showed mitotic figures, the dominating picture was that of atrophy and fibrosis. The process appeared to be one of long survival and of slow destruction of the grafts rather than one of successful transplantation.

From these experiments it may be concluded that local transplantation of this tumor cannot be accomplished by the methods used.[2]

However, from the examination of frogs which had been inoculated subcutaneously, intracranially, and intraabdominally, it became obvious that in a considerable number of them, tumors of the kidney had developed, and the proportion having kidney tumors became greater as the interval of time between inoculation and examination

[2] The fact that no local growth resulted at four different sites of inoculation may mean, of course, that none of these sites provided a suitable habitat for survival and multiplication of implanted tissue. Indeed it will be shown in a subsequent paper that successful local transplantation may be accomplished in the indifferent humors of the eye, and in the kidney itself, but not in the liver. In these experiments, even more strikingly than in the series here reported, there was evidence of selective affinity of the causal agent for a particular organ, for in over one-third of the frogs kidney tumors developed.

lengthened. Thus, in the animals examined within the first 3 months, the incidence of renal tumor corresponded with that observed in the control groups; but, in the next 3 months period, the incidence had become definitely greater among the experimental frogs, and decisively so in animals which survived inoculation more than 6 months. The results obtained with solid fragments and with cell suspensions were approximately the same. These findings are shown in Table I, in which are given, for each site of inoculation, the number of frogs examined at different intervals, and the number having kidney tumors.

TABLE I

Incidence of Kidney Tumors Developing in Frogs Inoculated with Living Tumor

Site of inoculation	Months after inoculation					
	0–3		4–6		Over 6	
Intramuscular..................	75	3	36	1	22	1
Subcutaneous..................	128	1	32	2	36	5
Intracranial..................	22	0	1	1	14	4
Intraabdominal................	89	0	33	5	78	17
Controls..................	683	16	166	10	104	7

In each column is given the number of frogs inoculated and (in bold face) the number having tumors. It is seen that, excepting the intramuscular inoculations, there is a rise in incidence after the initial 3 months periods. Approximately 20 per cent of the animals which had survived over 6 months had developed kidney tumors. However, in frogs which had been inoculated in the muscles, the frequency of renal tumors did not rise appreciably. In the bottom line is given the incidence in the control series. It is seen that rise in incidence is slight; the possible significance of this is discussed in the text.

The table is based upon a total of 566 frogs examined. It will be noted that in the intramuscular group but few were found to have kidney tumor. In the other three groups the incidence rose from less than 2 per cent during the first 3 months period to approximately 20 per cent in frogs which survived for more than 6 months. The tumors found resembled in every detail those occurring under natural conditions (Fig. 1). From these experiments it may be concluded that inoculation of living tumor at various sites, either as fragments or cell suspension, results in the development of a tumor only in that

organ in which it occurs spontaneously. The time required for devel-
opment of a kidney tumor appears to be, in most cases, at least 6
months.

Results with Desiccated and Glycerinated Tumor

234 frogs received intraabdominal injections of desiccates prepared
from 10 tumors, and 10 frogs were injected with an emulsion of a
glycerinated tumor. As shown below, the results obtained were
similar.

TABLE II

*Comparison of Percentage of Frogs Developing Kidney Tumor after Intraabdominal
Inoculation of Desiccated or Glycerinated Tumor, and of Living Tumor. The
Incidence in the Control Series Is Given in the Bottom Line*

Material		Months after inoculation		
		0–3	4–6	Over 6
Desiccated or glycer-inated tumor	Number of frogs examined	112	38	94
	Percentage having tumor	6.3	10.5	21.3
Living tumor	Number of frogs examined	89	33	78
	Percentage having tumor	0	15.2	21.8
Controls	Number of frogs examined	683	166	104
	Percentage having tumor	2.3	6.0	6.7

In inoculated animals surviving more than 6 months the results of the two
series are approximately alike.

In none of the animals did any tumors develop at the site of injec-
tion, or in any of the viscera within the celomic cavity. But, as in
the preceding series, there occurred a conspicuous increase in kidney
tumors (Figs. 2 to 4). These findings are detailed in Table II where is
given the number inoculated and the percentage having kidney tumor.
For comparison, the results obtained after intraabdominal inoculation
of living tumor are stated; in the bottom line, the incidence in the
controls is recorded. It will be noted that the kidney tumor occurred
in somewhat over 20 per cent of the frogs surviving injection with
desiccated or glycerinated material for a period of more than 6 months.

This result corresponds closely with values obtained after inoculating living tumor.

Of the 10 frogs which received an emulsion of glycerinated tumor, only 4 survived the initial 3 months period; in 2 of these frogs the kidney tumor was present. The results are then, quite similar with both types of material. At least in the case of the desiccates, which were used in the great majority of the experiments, it may justifiably be assumed that the material used for inoculation did not contain living tumor cells.

From these experiments the conclusion is warranted that the tumor-inducing agent can resist conditions incompatible with the viability of animal cells. When one considers all the evidence of the present paper, and that previously given (1), it seems more than likely that the agent is a virus.

Retransmission

The question whether tumors which developed after inoculation would be transmissible with greater ease than the original tumors was investigated. Unfortunately the mortality among the 84 frogs used was, by chance, very great during the initial 3 months periods. Thus, but a single group remained in which it was possible to evaluate the results, which, however, are of sufficient interest to report here.

The original tumor (designated 126) was a typical adenocarcinoma with many inclusions. A suspension of its cells was injected into the abdominal cavity of 18 frogs. Of these 9 survived for more than 6 months and 4 of them were found to have developed renal tumors. One of these tumors was of sufficient size to be palpable during life, and this was removed and inoculated into the abdominal cavity of 13 frogs. Histologically it resembled the original tumor, excepting that inclusions were present to so great an extent as to occupy in some regions nearly every nucleus. 2 of the 5 frogs which were examined 4 to 6 months after inoculation, as well as 2 of the 4 which survived for more than 6 months had renal tumors. Other details are given in Table III.

This experiment does not answer the question for which it was designed, but the results indicate that a strain of tumor-inducing agent which had a high virulence initially retained this property after passage.

Controls. Incidence of Spontaneous Tumor

Examination of 953 control frogs gave very different results from those of the experimental series. During the first 3 months period from the inception of the corresponding experiments, slightly over 2 per cent had renal tumors. This incidence rose slightly, to 6 per cent in the second 3 months period, and to 6.7 per cent in frogs surviving for more than 6 months (see Table II, bottom line). While this rise is far below the striking increase in the experimental groups, it may have considerable significance. There exists a real possibility that the neoplastic disease is transmissible from frog to frog. In captivity, frogs are of necessity maintained under more crowded conditions than exist in their natural environment, and confinement in the laboratory

TABLE III

Kidney Tumor Developing after Intraabdominal Inoculation of Fragments from Tumor 126, and after Inoculation of One of the Tumors Which Developed (R 126)

Tumor	Months after inoculation					
	0–3		4–6		Over 6	
126...............	8	**0**	1	**0**	9	**4**
R 126.............	4	**0**	5	**2**	4	**2**
Controls.........	12	**1**	6	**0**	4	**0**

The figures in the first column of each group express the number of inoculated or control animals; the bold face figures, the number having tumors.

would favor not only direct contact by also indirect transference of various agents. Experiments are now under way to test the possibility that the tumor-inducing agent may be transferred by means other than inoculation. However, from the fact that relatively few tumors were found among the control animals while a very considerable number occurred among the experimental frogs, the inference may be drawn that transference by "natural" means does not readily come about.

The incidence of tumors observed in the control series, and in those of the experimental animals surviving inoculation for less than 3 months, is of the same order as the incidence of spontaneous tumors. Examination of 10,317 frogs, most of them from students' physio-

logical and pharmacological laboratories, revealed that kidney tumor occurred in 2.7 per cent. It is interesting to note that while there was some variation in frequency in different lots, the incidence on the whole was quite constant and varied but little from year to year. It should be pointed out that frogs purchased from dealers are not usually freshly captured animals, but during the winter at least, have been in captivity for months. No seasonal variation in frequency of tumors was observed.

Inoculation of Foreign Species

A group of 44 frogs consisting of approximately equal numbers of *R. clamitans*, and half grown bullfrogs, *R. catesbiana*, received intra-abdominal injection of mixed desiccates from 2 tumors.

Solid fragments and cell suspension of 4 other tumors were inoculated, also intraabdominally, into 65 frogs of a subspecies of *R. pipiens* occurring in New Jersey. This breed differs from the New England species in coloration, and possibly in certain other characters.

None of these frogs of foreign species or alien breed developed the renal tumors, indicating species specificity of the causal agent.

COMMENTS

The experiments here reported all support the indication, first given by the frequent presence of intranuclear inclusions within the tumor cells, that the kidney tumor of leopard frogs is caused by a virus. Living tumor inoculated at various sites did not lead to local growth, but tumors developed in the kidney of approximately 20 per cent of frogs which survived for more than 6 months. Inoculation of desiccated tumor, and in one group of glycerinated tumor, led to similar results; in somewhat over 20 per cent of frogs which survived for more than 6 months, renal tumors occurred. On the other hand, during the first 3 months, less than 2 per cent of the inoculated frogs were found to have this neoplasm, an incidence quite similar to that obtained in the controls and for spontaneous tumors from examination of a large number of frogs. Attempts to transmit the tumor to alien species proved unsuccessful. These results can best be interpreted as indicating the existence in the inoculated material of an organ-specific carcinogenic agent having the attributes of a virus.

That certain viruses induce neoplastic proliferation is a well established fact. The problem of the etiologic relation of viruses to tumors and the nature of virus-induced tumors have been so recently and fully reviewed by Andrewes (5) and by Rous (6), that no further discussion is necessary here. But, it is worthy of emphasis that such tumors have proven to be true neoplasms, that several varieties of them are known to exist, and that they are not confined to one particular class of animals. The kidney tumor of frogs, which usually is carcinomatous in character, would appear to be another example. This tumor is of interest not only because of its probable etiology, but because its ready availability makes possible the study of the general characteristics of tumor growth in another and more primitive class of vertebrates.

SUMMARY

An epithelial tumor with acidophilic intranuclear inclusions frequently occurs in the kidneys of leopard frogs. This tumor usually has the appearance of an infiltrating and destructive adenocarcinoma, which, when large, not uncommonly metastasizes; less often it is more orderly and adenomatous.

When inoculated as living fragments or cell suspensions into the lymph sacs, the cranial cavity, or the abdomen, no significant local growth results and the implanted material is resorbed. However, in approximately 20 per cent of the frogs surviving inoculation for more than 6 months, tumors develop in the kidney, which are like the "spontaneous" neoplasms. The incidence far exceeds that in the controls.

Desiccated and glycerinated tumor injected into the abdomen gives the same result as inoculation with living tumor; in somewhat over 20 per cent of animals surviving more than 6 months kidney tumors occur.

In alien species of frogs, no such tumors are produced by inoculation either with living or with desiccated tumor.

These experiments indicate the probability that the kidney tumor of the leopard frog is caused by an inclusion-forming, organ-specific virus.

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EXPLANATION OF PLATE 15

All sections were stained with hematoxylin and phloxin.

FIG. 1. Adenocarcinoma of the kidney found 176 days after intracranial inoculation with fragments of living tumor. The general character is like that of the spontaneous tumors. × 100.

FIG. 2. Adenocarcinoma of the kidney which developed after intraperitoneal injection of desiccated tumor. 355 days after inoculation, tumors were found in both kidneys; they had fused to form a large mass measuring 23 x 10 x 6 mm. Several metastatic nodules were located in the liver, and, on microscopic examination, tumor emboli were observed in some of the intrahepatic veins. × 100.

FIG. 3. Extension of tumor cells into the lumen of a thin walled vessel of the tumor shown in Fig. 2. The group of tumor cells is indicated by an arrow. × 300.

FIG. 4. A tumor cell embolus, also from the specimen shown in Fig. 2, is blocking a small intrahepatic vein. × 436.

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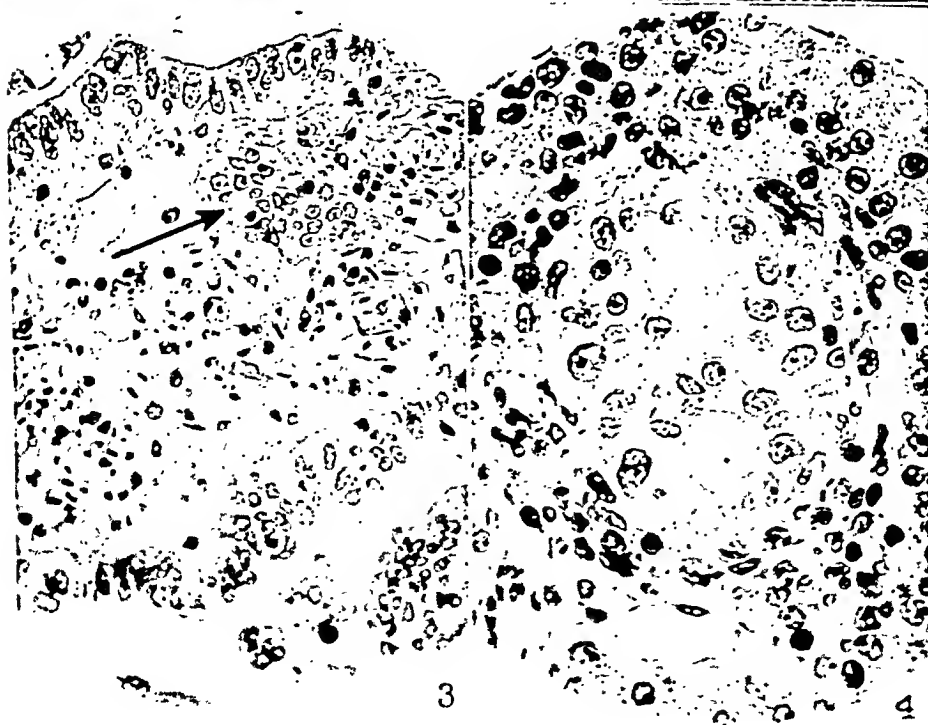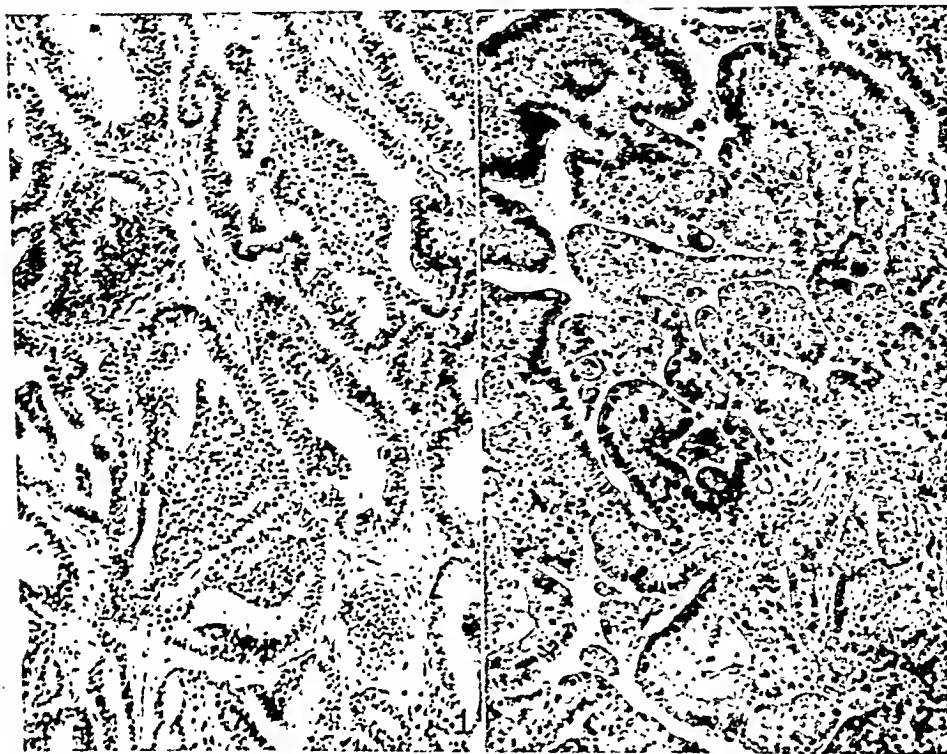
CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

XII. The Immunological Properties of an Artificial Antigen Containing Cellobiuronic Acid

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The problem of understanding the factors which govern the immunological specificity of bacterial polysaccharides is essentially biochemical in nature and can be approached in two ways. The chemical constitution of these complex substances may be elucidated by the classical methods of organic chemistry in the hope of correlating differences in structure with changes in specificity. On the other hand an approach may be made by rendering simple carbohydrates of known constitution antigenic through combination with protein, and correlating the specificity of the antibodies ⸱elicited with known changes in the chemical structure of the carbohydrate radicals in question. Although the latter method has certain obvious limitations, in our initial chemo-immunological studies on the specificity of carbohydrates we chose the second mode of approach, for it was our opinion that without a far reaching biological understanding, a purely chemical interpretation of this important immunological problem would be sterile indeed.

From initial attempts to understand the factors underlying the immunological specificity of carbohydrates certain fundamental facts have been revealed through the application of the immunochemical approach. It has been possible to demonstrate that intra- and intermolecular differences in the configuration of mono- and disaccharides are influential in determining specificity (1). Likewise it has been found that the introduction of a labile grouping, such as the acetyl group, will alter the immunological specificity of a monosaccharide (2). More recently it was demonstrated that the conversion of the

primary alcohol grouping occupying the sixth position in a hexose to the carboxyl group, conveys a new and distinct specificity upon the saccharide in question (3).   Artificial antigens containing glucuronic and galacturonic acids, as opposed to those containing the corresponding monosaccharides, assume a new and important biological property, namely the capacity to precipitate in antipneumococcal sera (4).   Although the hexose-uronic acid antigens possess serological properties which correlate them with the bacterial polysaccharides, it has not as yet been possible to induce immunity to pneumococcal infection in experimental animals by immunization with glucuronic or galacturonic acid antigens.   Therefore, in order to understand more fully the rôle which the aldobionic acids, the fundamental building stones of certain bacterial polysaccharides, play in immunological phenomena we have found it advisable to study the immunological properties of antigens containing these acids.

The aldobionic acid, cellobiuronic acid, is a disaccharide constituted from one molecule of glucuronic acid combined in glucuronosidic linkage with one molecule of glucose, through the hydroxyl group of the fourth carbon atom of the hexose. This linkage has the β configuration, and the ring structure of the two hexose constituents have been proven to be that of a pyranoside (5).   Cellobiuronic acid can be obtained as an amorphous substance from the hydrolysis products of the capsular polysaccharides of either Types III or VIII Pneumococcus (6).   The amorphous disaccharide can be converted to a crystalline alkaloidal salt, or to a characteristic crystalline heptaacetyl methyl ester.   The latter derivative has served as the source material for the synthesis of the derivatives used in the present investigation.

Cellobiose is, of course, the disaccharide obtained from the hydrolysis of cellulose.   It is constituted from one molecule of glucopyranose joined in glucosidic linkage to the hydroxyl group of a second glucopyranose molecule on carbon atom 4.   The configurational relationship of all the asymmetric carbon atoms of cellobiose and cellobiuronic acid is identical, as is that of the intramolecular linkage. The p-aminobenzyl glycosides of cellobiose and cellobiuronic acid both have the β configuration.   The only difference in these two substances, therefore, is the grouping occupying the twelfth position, which in the case of cellobiose is a primary alcohol group (CH$_2$OH) and in cellobiuronic acid a carboxyl group (COOH).   Any differences in the immunological properties of antigens containing these two saccharides may therefore be attributed to this difference in chemical constitution. For the purpose of comparison the serological properties of two additional antigens, one containing the azobenzyl glycoside of glucose and the other that of glucuronic acid, have been included in this study.   The structural relationship of these four

glycosides can be seen from the following graphic formulae in which R represents the aglucon —$CH_2C_6H_4NH_2$.
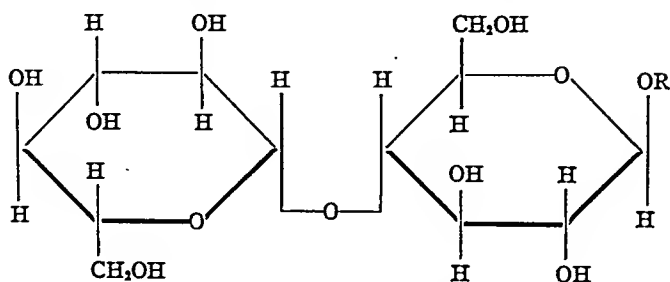
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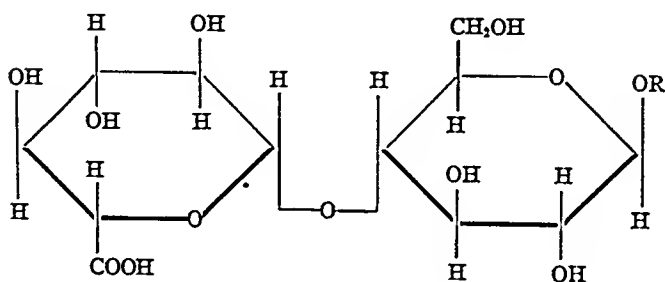
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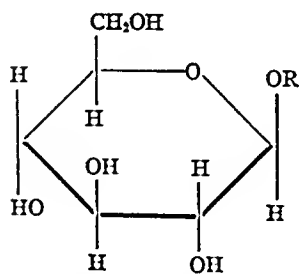
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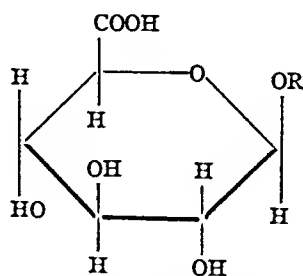
p-aminobenzyl β-glucuronide

Chemical Methods

Hexaacetyl p-Nitrobenzyl β-Cellobioside.—22 gm. of acetobromo cellobiose (7) were dissolved in 75 cc. of anhydrous chloroform. 6.6 gm. of dry *p*-nitrobenzyl alcohol and 7.2 gm. of dry silver oxide were added. The mixture was shaken until the supernatant liquid no longer gave a test for the bromo derivative. After filtering and removing the chloroform *in vacuo* the glycoside crystallized on the

addition of ethyl alcohol.   10 gm. of crude glycoside were recovered.   The substance was recrystallized several times from alcohol yielding 7.6 gm.   The pure substance was obtained as glistening needles melting at 178–180° (uncorrected).

Rotation.—$[\alpha]_D^{22°} = -34.8°$ in $CHCl_3$ (C = 1 per cent).

Analysis.—$C_{33}H_{41}O_{20}N$.   Calculated.   C 51.4, H 5.4.
                                   Found.   C 51.3, H 5.6.

p-Nitrobenzyl β-Cellobioside.—2.5 gm. of hexaacetyl *p*-nitrobenzyl cellobioside were suspended in 50 cc. of methyl alcohol and deacetylated at 0° with 1/30 mole of barium methylate (8).   The barium was removed by adding one equivalent of N/1 sulfuric acid.   After filtering off the precipitated barium sulfate, the crude deacetylated glycoside was obtained by concentrating the filtrate *in vacuo*, and recrystallizing the residue from the minimum quantity of 90 per cent ethyl alcohol; 1.2 gm. were recovered.   The glycoside was repeatedly crystallized from this solvent until the optical rotation remained constant.   The compound was obtained as rosettes of needles melting at 199–200° (uncorrected).

Rotation.—$[\alpha]_D^{20°} = -32.3°$ in H_2O (C = 1 per cent).

Analysis.—$C_{19}H_{27}O_{13}N$.   Calculated.   N 2.93.
                                   Found.   N 2.84.

p-Aminobenzyl β-Cellobioside.—1.0 gm. of *p*-nitrobenzyl cellobioside was dissolved in 50 cc. of methyl alcohol and reduced catalytically with hydrogen and platinum oxide as catalyst (9).   On concentrating the alcoholic solution to a syrup *in vacuo*, and taking up in 95 per cent ethyl alcohol, 0.9 gm. of the glycoside was obtained as fine white needles melting at 188–190° (uncorrected) with decomposition.

Rotation.—$[\alpha]_D^{22°} = -35.2°$ in H_2O (C = 0.5 per cent).

Analysis.—$C_{19}H_{29}O_{11}N$.   Calculated.   N 3.13.
                                   Found.   N 3.03.

p-Nitrobenzyl β-Glycoside of Hexaacetyl Cellobiuronic Acid Methyl Ester.—11.9 gm. of acetobromo cellobiuronic acid methyl ester (10) were dissolved in 60 cc. of dry chloroform, and 5.2 gm. of *p*-nitrobenzyl alcohol and 6 gm. of dry silver oxide added.   The mixture was shaken for 4 hours, and the chloroform solution finally separated from the residue of silver salts by filtration.   The chloroform was removed *in vacuo*, and on dissolving the oily residue in ethyl alcohol, the crude glycoside crystallized.   6.2 gm. of an impure product melting between 160° and 180° were obtained.   This substance was now dissolved in 300 cc. of hot ethyl alcohol and after standing 1 hour at room temperature 3.8 gm. of the hexaacetyl *p*-nitrobenzyl glycoside of cellobiuronic acid methyl ester were obtained as glistening pale yellow crystals melting at 192–193°.

Rotation.—$[\alpha]_D^{20°} = -41.7°$ in $CHCl_3$ (C = 0.6 per cent).

Analysis.—$C_{30}H_{36}O_{18}N(COOCH_3)$.   Calculated.   C 50.7, H 5.2, OCH_3 4.1.
                                   Found.   C 50.4, H 5.1, OCH_3 4.1.

p-Nitrobenzyl β-Glycoside of Cellobiuronic Acid Methyl Ester.—3.5 gm. of the acetylated glycoside were suspended in 150 cc. of methyl alcohol and deacetylated at 0° with barium methylate exactly as was the corresponding cellobioside.   After

removal of the barium, 1.6 gm. of pure glycoside melting at 188–189° were obtained from the mother liquors. The compound crystallized as beautiful rosettes of needles from ethyl alcohol.

Rotation.—$[\alpha]_D^{22°} = -48.1°$ in CH_3OH (C = 1 per cent).

Analysis.—$C_{20}H_{27}O_{14}N$. Calculated. OCH_3 5.96.

Found. OCH_3 6.14.

Barium Salt of p-Aminobenzyl β-Glycoside of Cellobiuronic Acid.—1 gm. of the nitrobenzyl glycoside of cellobiuronic acid methyl ester was converted to the amino compound by catalytic reduction. The colorless alcoholic solution was evaporated *in vacuo*, the glycoside taken up in a few cubic centimeters of water, and one equivalent of 0.4 N barium hydroxide was slowly added. The mixture was warmed to 50° to effect complete hydrolysis of the methyl ester group. The clear very pale yellow solution of glycoside was concentrated to small volume *in vacuo*, and then poured into 20 volumes of chilled absolute ethyl alcohol. 0.9 gm. of the barium salt of the *p*-aminobenzyl glycoside of cellobiuronic acid was isolated as an amorphous powder, readily soluble in water and insoluble in the usual organic solvents.

Rotation.—$[\alpha]_D^{22°} = -44.0°$ in H_2O (C = 0.5 per cent).

Analysis.—$C_{19}H_{25}O_{12}N\frac{1}{2}Ba$. Calculated. N 2.66, Ba 13.04.

Found. N 2.65, Ba 12.50.

p-Aminobenzyl β-Glucoside and β-Glucuronide.—These derivatives were prepared by methods previously described (1,3).

Immunological Reactions
Methods

Immunizing antigens were prepared by combining the diazotized *p*-aminobenzyl β-glycoside of glucose, glucuronic acid, cellobiose, and cellobiuronic acid with the globulin fraction of normal horse serum. For each 600 mg. of globulin was used 1 millimole of glycoside. Before diazotization the barium salt of the cellobiuronide was first converted to the sodium salt by the addition of 1.2 equivalents of sodium sulfate, followed by removal of precipitated barium sulfate. The technique of preparing the antigens was the same as that described in earlier studies, as was the intravenous immunization of the rabbits. Two to three courses were given consisting of six daily intravenous injections of 5 mg. of antigen dissolved in sterile physiological salt solution. 7 days after the last injection the animals were bled from the ear, and the serum collected in the usual fashion.

In order to avoid protein cross reactions the test antigens used in the serological analysis were prepared by combining the diazonium derivatives of the various glycosides to the protein of chicken serum. The test antigen containing the azobenzyl ether of the capsular polysaccharide of Pneumococcus Type III was prepared by combining its diazonium derivative to a slight excess of crystalline egg albumin (11). Instead of precipitating the coupled derivative at the isoelectric point, the alkaline reaction product was dialyzed at 0° for 24 hours. The pH of

the solution was adjusted to 7.5, and the salt content made to 1 per cent by the addition of solid NaCl. In this way a soluble protein-polysaccharide antigen was obtained which appeared to be free from the uncombined polysaccharide derivative.

The immunizing antigens are referred to in the tables as C-globulin (cellobiose-globulin), Ca-globulin (cellobiuronic acid-globulin), G-globulin (glucose-globulin), and Ga-globulin (glucuronic acid-globulin). The test antigens prepared by combining the diazotized *p*-aminobenzyl glycosides to chicken serum, are referred to as C-chick, Ca-chick, etc. The S III test antigen is referred to as S III-egg, and the various glycosides are designated as C, Ca, G, and Ga for the cellobioside, cellobiuronide, glucoside, and glucuronide respectively. The technique of the specific inhibition tests is the same as that described in earlier studies.

1. Precipitin Reactions

Antisera of Rabbits Immunized with Antigens Containing the Azo-benzyl Glycosides.—The sera of rabbits immunized with azoprotein (horse globulin) antigens containing the four saccharides, glucose (G), glucuronic acid (Ga), cellobiose (C), and cellobiuronic acid (Ca), yield a marked precipitate when the homologous carbohydrate derivative combined with a heterologous protein (chicken serum) is added. An analysis of Table I reveals, however, that the antibodies elicited by the glucose, glucuronic acid, and cellobiose antigens show sharper specificity than do those elicited by the antigen containing cellobiuronic acid. Cellobiose is a disaccharide constituted from one molecule of glucose combined in β-glucosidic union to the fourth carbon atom of a second glucose molecule. It is not surprising, therefore, that an antiserum elicited by an antigen containing this disaccharide would react with a test antigen containing the monosaccharide glucose and *vice versa*.

It has been shown that antigens containing glucose and glucuronic acid give rise to antibodies which are sharply specific and show no serological crossing (3). The fact that neither the glucuronic nor the cellobiuronic acid antigens react in C or Ca antisera lends additional support to the view that the conversion of the primary alcohol grouping (CH₂OH) of a saccharide to the carboxyl group (COOH) confers upon the newly formed uronic acid antigen a new and distinct specificity. Thus it is interesting to note that an antiserum to Ga-globulin, in addition to precipitating the homologous test antigen, likewise precipitates the test antigen containing cellobiuronic acid, but not the corresponding cellobiose antigen, and of course not the glucose antigen.

The lack of specificity exhibited by the cellobiuronic acid antiserum is difficult to explain. In a previous study on the specificity of artificial carbohydrate-protein antigens containing disaccharides (1 b) it was found that the specificity of antibodies elicited by such antigens was directed not only toward the disaccharide molecule as a whole, but toward the terminal hexose molecule as well, and but little, if at all, toward the hexose molecule bearing the aglucon. Thus an antiserum

TABLE I

Homologous and Heterologous Precipitin Reactions of Glucose, Glucuronic Acid, Cellobiose, and Cellobiuronic Acid Antisera

Antiserum prepared by immunization with	Test antigen used	Final dilution of test antigen			
		1:5,000	1:10,000	1:25,000	1:50,000
Ca-Globulin	Ca-chick	++	+++±	++±	++±
	C-chick	++±	++	++	+±
	Ga-chick	++	++	+±	+
	G-chick	+±	+	+	+
C-Globulin	Ca-chick	0	0	0	0
	C-chick	+++	+++	++±	++
	Ga-chick	0	0	0	0
	G-chick	+±	++	+±	+
Ga-Globulin	Ca-chick	++	++	++	+±
	C-chick	0	0	0	0
	Ga-chick	+++	+++	++±	++
	G-chick	0	0	0	0
G-Globulin	Ca-chick	0	0	0	0
	C-chick	++±	++±	++	+±
	Ga-chick	0	0	0	0
	G-chick	+++	+++	++±	+±

to an azoprotein containing lactose (4 β-glucosido-galactose) reacted with a test antigen containing galactose but not with one containing glucose. In the antiserum prepared by immunization with cellobiuronic acid antigen (4 β-glucuronosido-glucose) however, one finds antibodies capable of reacting not only with the glucuronic acid test antigen but with test antigens containing glucose and cellobiose as well. It appears, therefore, that in the serum to cellobiuronic acid there

is a reflection not only of the aldobionic acid molecule as a whole, but there are antibodies capable of reacting with both individual constituents, glucose and glucuronic acid, as well.

Reactions of a Test Antigen Containing the Capsular Polysaccharide of Pneumococcus Type III.—The capsular polysaccharide of the Type III Pneumococcus is constituted from molecules of cellobiuronic acid linked in glycosidic union to form a large non-diffusible molecule the precise magnitude of which has not been ascertained. On hydrolysis of the polysaccharide by dilute mineral acid, cellobiuronic acid is obtained. There is certain evidence in support of the view that units of aldobionic acid of sufficient molecular size (di- and tetra-aldobionides) still retain the property of precipitating in homologous Type III antipneumococcus horse serum (12). It has occurred to us, therefore, that

TABLE II

Precipitin Reactions of Pneumococcus Type III Polysaccharide Test Antigen in Glucose, Cellobiose, Glucuronic Acid, and Cellobiuronic Acid Antisera

Antisera prepared by immunization with	Final dilution of S III antigen				
	1:5,000	1:10,000	1:25,000	1:50,000	1:200,000
Ca-globulin....................	+++	+++	++±	++	+
C-globulin....................	0	0	0	0	0
G-globulin....................	0	0	0	0	0
Ga-globulin....................	0	0	0	0	0

the capsular polysaccharide of Type III Pneumococcus, or the monoaminobenzyl ether of this substance either free or in combination with a protein, might react in cellobiuronic acid antiserum. That this is the case and that the reaction takes place only in cellobiuronic acid antiserum, and not in cellobiose, glucose, and glucuronic acid antisera, can be seen from the results given in Table II. The significance of these findings will be discussed later.

2. Inhibition Reactions

1. Cellobiuronic Acid Antiserum.—The specificity of the cross precipitation reactions of Ca, C, Ga, G, and S III test antigens in cellobiuronic acid antiserum may best be studied by means of the specific inhibition tests, the results of which are given in Table III. From

the results presented in Table III it is seen that the reaction between cellobiuronic acid antiserum and homologous antigen is specific, for it can be inhibited only by the homologous glycoside, Ca. The heterologous reactions of the hexose and disaccharide antigens, C and G, on the other hand, are inhibited by the cellobiuronide, the glucoside, and cellobioside respectively. It is interesting to observe that the reaction of C antigen in Ca antiserum is inhibited only by Ca and C and not by G, whereas the reaction of the G antigen is inhibited not only by Ca and G, but by the cellobioside C as well. The fact that the cellobioside inhibits the reaction of the monosaccharide antigen G and that the reverse is not true, indicates that the precipitation of C antigen in Ca antiserum approaches more closely the homologous reac-

TABLE III

Inhibition of Precipitins in Cellobiuronic Acid Antiserum by Homologous and Heterologous Glycosides

Inhibiting glycoside	Cellobiuronic acid antiserum				
	Test antigen 1:10,000				
	Ca-chick	C-chick	Ga-chick	G-chick	S III chick
Ca.............	0	0	0	0	0
C.............	++	0	+±	0	+++±
Ga.............	++	+±	0	+	0
G.............	+++	++	+±	0	+++±
None...........	+++	++±	++	++	+++

tion than does the G reaction. It is of further interest to observe that the glucuronide Ga does not inhibit the reaction of C or G antigens in Ca antiserum. This fact would indicate that a uronic acid antibody *per se* is not involved in the precipitation reaction of the antigens in question. It likewise appears that the precipitation of both Ga and S III antigens in Ca antiserum involves specifically only a uronic acid antibody, for both reactions are inhibited not by the glucoside and cellobioside, but only by the glucuronide and cellobiuronide.

2. *Glucose, Glucuronic Acid, and Cellobiose Antisera.*—The specific inhibition tests of various antigens in G, Ga, and C antisera are given in Tables IV, V, and VI and the interpretation of the specificity of the

different tests presents no difficulty.   In each instance the homologous reaction is inhibited only by the homologous glycoside, whereas the heterologous reaction is inhibited both by the glycoside homologous to the antiserum as well as by that homologous to the antigen.   From these tests one important and new fact is brought to light, namely that the antibodies elicited by the uronic acid derivatives of glucose

TABLE IV

Inhibition of Precipitins in Cellobiose Antiserum by Homologous and Heterologous Glycosides

| Inhibiting glycoside | Cellobiose antiserum | |
| | Test antigen 1:10,000 | |
	C-chick	G-chick
C.................	0	0
G.................	+++±	0
Ca................	+++±	+++
Ga................	+++±	+++
None..............	+++±	+++

TABLE V

Inhibition of Precipitins in Glucose Antiserum by Homologous and Heterologous Glycosides

| Inhibiting glycoside | Glucose antiserum | |
| | Test antigen 1:10,000 | |
	G-chick	C-chick
G.................	0	0
C.................	++±	0
Ca................	+++	++
Ga................	+++	++
None..............	+++	++

TABLE VI

Inhibition of Precipitins in Glucuronic Acid Antiserum by Homologous and Heterologous Glycosides

| Inhibiting glycoside | Glucuronic acid antiserum | |
| | Test antigen 1:10,000 | |
	Ga-chick	Ca-chick
Ga.....................	0	0
Ca.....................	++	0
G......................	++	+±
C......................	++±	+±
None...................	+++	++

and cellobiose, glucuronic and cellobiuronic acids, are distinct and specific.   A similar relationship has been established in a previous study for antigens containing the azophenol glycosides of the mono-saccharide glucose and the disaccharide cellobiose (1b) and is now reaffirmed for antigens containing the azobenzyl glycosides of these saccharides.

3. Precipitin Reactions of Antipneumococcus Sera with Test Antigens Containing the Azobenzyl Glycosides.—In a previous communication (3) it was shown that an azoprotein antigen containing glucuronic acid precipitates in antipneumococcus horse sera Types II, III, and VIII, whereas the corresponding glucose antigen is serologically inert. The precipitin reactions of Ga, C, and Ca test antigens in antipneumococcus horse and rabbit sera are presented in Table VII. It can be seen that the antigen containing the disaccharide cellobiose reacts not at

TABLE VII

Precipitin Reactions of Cellobiose, Cellobiuronic Acid, and S III Antigens in Antipneumococcus Sera

Anti-pneumo-coccus serum Type	Test antigen used	Antipneumococcus horse serum				Antipneumococcus rabbit serum			
		Final dilution of test antigen				Final dilution of test antigen			
		1:10,000	1:50,000	1:250,-000	1:1,000,-000	1:10,000	1:50,000	1:250,-000	1:1,000,-000
I	C-chick	0	0	0	0	—	—	—	—
II	"	+	0	0	0	0	0	0	0
III	"	+±	+	±	0	±	±	0	0
VIII	"	+++±	++	+	0	++	+	+	±
I	Ca-chick	0	0	0	0	—	—	—	—
II	"	++++	+++	++	+	+±	+	+	±
III	"	++++	+++±	++±	+	++±	++±	++	+
VIII	"	++±	+++±	++	+	+±	+	±	0
I	S III-egg	0	0	0	0	—	—	—	—
II	"	++++	++±	+	0	0	0	0	0
III	"	++++	+++	++	+	++++	++++	++	+
VIII	"	+++	++±	+±	+	+	+	+±	±

all in Type I antipneumococcus horse serum, only feebly in Type II serum, and slightly more vigorously in Type III serum, whereas in Type VIII serum the reaction is still detectable in dilutions of one part in a quarter million. In rabbit sera of the corresponding types the reactions are negligible save in Type VIII serum. The chemical constitution of the Type VIII capsular polysaccharide is as yet unknown, but it appears to be constituted from molecules of glucose and glucuronic acid in the ratio of 7:2 (6a). On hydrolysis there is obtained glucose

and cellobiuronic acid. Whether the affinity of the cellobiose antigen for part of the Type VIII antibody is indicative of a cellobiose residue or configuration, in this polysaccharide molecule cannot be answered at the present time. The great avidity of the cellobiuronic acid antigen, on the other hand, for the antibodies of Types II, III, and VIII antipneumococcus sera, both of the rabbit and the horse, is evident from the results given in Table VII. It has been found that the capsular polysaccharides of both Types III and VIII contain cello-

TABLE VIII

Inhibition of Precipitin Reactions of Cellobiuronic Acid and S III Antigens in Antipneumococcus Horse Sera Types II, III, and VIII

Antipneumococcus horse serum Type	Inhibiting glycoside	Test antigen 1:10,000	
		Ca-chick	S III-egg
II	Ca	0	0
	C	+++±	+++
	Ga	±	0
	G	+++±	++±
	None	++++	+++
III	Ca	0	++++
	C	++++	++++
	Ga	+++±	++++
	G	++++	++++
	None	++++	++++
VIII	Ca	0	0
	C	++	+±
	Ga	+	+
	G	++	+±
	None	++	++±

biuronic acid as an integral part of the carbohydrate molecule. It is therefore not surprising that the uronic acid antigen reacts in these antisera. In the case of the test antigen containing the azobenzyl ether of the capsular polysaccharide of Type III Pneumococcus, the reaction in both Types III and VIII antisera is to be expected. However, the precipitation of this antigen in antipneumococcus serum Type II is surprising, particularly in view of the established specificity of the Type III pneumococcus polysaccharide itself. This remarkable

acquired property of the Type III capsular polysaccharide, when in combination with a large protein molecule, is difficult to understand. Control tests in which the p-aminobenzyl ether of the Type III polysaccharide itself was added to Type II antipneumococcus serum, showed no reaction whatsoever, nor did comparable dilutions of uncoupled egg albumin react in Type II serum. Qualitative tests indicate that the capsular polysaccharide of Type II Pneumococcus contains a uronic acid. That a reflection of this constituent is found in homologous antiserum is evident from the serological tests in which both glucuronic and cellobiuronic acid test antigens are found to react in high dilution. The explanation of the precipitation of the S III—egg antigen in Type II serum, and the failure of the uncombined capsular Type III polysaccharide to react may reside in the comparative molecular magnitude of the two substances in question. However, from the results of the specific inhibition tests given in Table VIII it is evident that precipitation of the S III antigen in antipneumococcus horse serum Type II is inhibited both by the glucuronide and the cellobiuronide, whereas in Type III serum the reaction is inhibited by neither glycoside. In Type VIII serum, on the other hand, inhibition is caused by the cellobiuronide, and precipitation is greatly diminished in the presence of glucuronide, though not completely prevented. It appears, therefore, that in Type II serum at least a portion of the antibody is directed toward the uronic acid, and when the heterologous S III test antigen is added to the immune serum union occurs between this antibody and the glucuronic acid portion of the test antigen. This reaction may be completely inhibited even by the simple glucuronide. In Type III antiserum the antibodies obviously combine with the polysaccharide molecule as a whole, for the reaction between the S III antigen and antiserum cannot be inhibited by glucuronide or cellobiuronide. In Type VIII serum, on the other hand, the reaction is inhibited by the cellobiuronide and not entirely by the glucuronide, a result which is to be anticipated on account of the common cellobiuronic acid nucleus present in the Type III and VIII polysaccharides.

The precipitation of the cellobiuronic acid antigen in Types II, III, and VIII sera is in each instance inhibited by cellobiuronide. But only in Type II serum is precipitation in these sera inhibited by

glucuronide, a fact which further substantiates the hypothesis that the reactive antibody in Type II serum is directed toward the hexuronic rather than the aldobionic acid. In conclusion it should be emphasized that the S III antigen (Table VII) does not react in Type II antipneumococcus rabbit serum. This fact indicates that an antibody directed against the uronic acid constituent is not present in antipneumococcus rabbit serum and confirms earlier observations (3,4).

DISCUSSION

The functional rôle of acid groups in determining certain of the serological characteristics of bacterial polysaccharides, a possibility which was foreseen some years ago by Landsteiner (13) from his work on azoprotein antigens, is affirmed by the results of the present study. The sharp specificity exhibited by antigens containing glucose and glucuronic acid is not shared by similar antigens containing the disaccharide cellobiose and its derivative cellobiuronic acid. The antiserum elicited by the cellobiuronic acid antigen reacts not only with the disaccharide antigen, but with simple glucose and glucuronic acid antigens as well. Thus it appears that the antibody to which cellobiuronic acid gives rise is directed toward both molecular constituents as well as toward the molecule as a whole. The hexose uronic acid constituents of the intact capsular polysaccharides in the form in which they occur in the bacterial cells are obviously not the sole determinant in orienting the specificity of the antipolysaccharide immune body. Nor can this property be attributed to the aldobionic acid molecule alone, for if it were the cellobiuronide should inhibit the reaction of Type III polysaccharide in homologous antiserum. That the uronic acids account for serological cross reactions, however, cannot be denied from the results of the foregoing experiments. The carbohydrate antibodies in antipneumococcus serum may be regarded as a mixture of different but closely related proteins certain of which are reactive with artificial antigens containing the simpler saccharide components of the capsular polysaccharide, but all of which are removed by absorption with the homologous type specific carbohydrate.

The capsular polysaccharide of Type III Pneumococcus appears to be a macro molecule constituted from units of cellobiuronic acid linked

in glycosidic union. The position of linkage, a problem in which we in this laboratory are at present engaged, is as yet undetermined. The molecular magnitude of the Type III carbohydrate is likewise uncertain (14). Yet in comparison to a protein molecule, the pneumococcus polysaccharide is a relatively simple chemical entity, for it is a molecule in which the same fundamental unit, cellobiuronic acid, is repeated periodically over and over again. One might anticipate, therefore, that an antibody elicited by cellobiuronic acid should bear some serological relationship to the parent polysaccharide itself. That this is the case is evident from the results of the foregoing serological analysis. A serum to the cellobiuronic acid antigen precipitates the capsular polysaccharide of Type III Pneumococcus when the latter is combined with egg albumin. Although it has not been indicated in the protocols, both the free capsular polysaccharide and the uncombined aminobenzyl ether of the latter likewise precipitate feebly in cellobiuronic acid antiserum in dilutions as high as one part in a quarter million. The antiserum to cellobiose, on the other hand, shows none of these precipitation reactions. The results of this study indicate, therefore, that the aldobionic acids have a unique biological function in determining the immunological characteristics of certain encapsulated microorganisms.

In conclusion it can be said that the antiserum to the artificial antigen containing cellobiuronic acid conveys passive protection on mice to infection with virulent pneumococci Types II, III, and VIII. The results of these experiments will be reported in a later communication.

SUMMARY

1. Artificial antigens containing the azobenzyl glycosides of the disaccharide cellobiose and the aldobionic acid, cellobiuronic acid, give rise in rabbits to antibodies which are specific and characteristic of the saccharide constituent. The antiserum to cellobiuronic acid shows broader serological cross reactions than does that to cellobiose.

2. An antiserum to the cellobiuronic acid antigen precipitates the capsular polysaccharide of Type III Pneumococcus when the latter is combined with a heterologous protein.

3. The cellobiuronic acid test antigen precipitates vigorously in antipneumococcus sera Types II, III, and VIII. The mechanism of these reactions is discussed.

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RENAL INSUFFICIENCY FOLLOWING TRYPSIN INJECTION INTO THE RENAL ARTERIES*

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PLATE 16

For many years, it has been accepted that derangement of kidney function is intimately related to hypertension. Fishberg (1), de Wesselow (2) and Volhard (3) have pointed out that, with the exception of amyloid degeneration of the kidneys, severe kidney derangement is consistently accompanied by hypertension. The frequent absence of hypertension in amyloid involvement of the kidney has been supposed to be due to the cachexia accompanying this disease.

However, the direct proof of the relationship of deranged kidney function to hypertension is still lacking in the patient. Carrière *et al.* (4), Kylin (5), Brems (6), Loewenstein (7), Bohn (8), Hülse and Strauss (9) and Major (10) have reported the presence of abnormalities in the chemical composition of the blood in patients suffering with hypertension. These results were not substantiated by Page (11), Elliott and Nuzum (12), Weinstein and Weiss (13), Leiter (14), de Wesselow and Griffiths (15), Aitken and Wilson (16), Jackson *et al.* (17) and Andes *et al.* (18), who have reported that the blood of patients suffering with hypertension is apparently normal except when marked kidney excretory insufficiency is present. Nor could these workers establish the presence of abnormal pressor substances. The inability to demonstrate conclusively any qualitative or quantitative change in the blood of hypertensive patients who have kidney function essentially normal does not disprove the possibility that hypertension is related to derangement in the kidney, but it does tend to deter the facile assumption that this relationship has been demonstrated clinically.

Experimental studies upon laboratory animals also fail to give any clear indication of a relationship between kidney function and hypertension. Pässler and Heineke (19), Chanutin and Ferris (20), and Wood and Ethridge (21) have reported hypertension following the surgical removal of such large amounts of normal kidney tissue as to cause renal excretory insufficiency. However, Anderson (22) found excretory insufficiency alone, without hypertension, after a similar procedure. Cash (23) on the other hand observed the occurrence of hypertension following partial nephrectomy and renal artery ligation leading to kidney necrosis and renal excretory insufficiency. Arnott and Kellar (24) observed hypertension

with concomitant renal insufficiency following the repeated administration of sodium oxalate, although their results could not be confirmed by Scarff and McGeorge (25). Hartman *et al.* (26) observed hypertension to result from repeated irradiation of the kidney, sufficient to lead to severe renal excretory insufficiency. Apfelbach and Jensen (27) could detect no hypertension in their animals following the injection of charcoal into both renal arteries with consequent severe renal excretory insufficiency. Finally, Goldblatt *et al.* (28) have reported that hypertension occurred following the production of either unilateral or bilateral renal ischemia. This last work has been confirmed by numerous workers, but the mechanism responsible for this hypertension is still unknown. Apparently this hypertension can occur following renal ischemia without gross evidence of renal excretory insufficiency, although there is little doubt that the type of hypertension produced in this manner can be masked by the presence of normal kidney tissue (29). The frequent temporary retention of blood nitrogen products immediately following bilateral renal artery clamping indicates that the renal excretory efficiency does suffer, at least temporarily.

This brief review makes it apparent that there is no agreement concerning the relationship between renal excretory insufficiency and hypertension or that between renal parenchymal damage and hypertension. In an effort to determine the interrelation of these three variables, renal excretory insufficiency, renal parenchymal damage and hypertension, a study was made of the effect of injection of trypsin into the arterial supply of the kidney of normal dogs. Trypsin was tried because, according to Rich and Duff (30), it produced hemorrhagic necrosis and rapid arteriolar hyalinization when injected subcutaneously, and preliminary experiments showed that trypsin injected into the renal artery produced renal damage. In addition, a study was made of the effects of renal ischemia produced by the Goldblatt clamp on kidneys previously damaged by trypsin injection and another of the effects of renal ischemia of one kidney in the presence of damage to the second produced by trypsin. It was hoped in this way that it would be possible to evaluate more clearly the interrelation of renal hypertension to renal parenchymal damage and renal excretory insufficiency.

Methods

(a) *The Injection of Trypsin Solution into the Renal Artery.*—A purified beef extract was the source of the trypsin. A concentrated solution of this was procured.[1] On the day of use, a 1 per cent suspension was prepared in normal saline,

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[1] Procured from the Wilson Laboratories through the kindness of Dr. D. Klein.

shaken strongly for 15 minutes, filtered through paper once and then Berkefeld filtered. Just before injection, the solution was warmed to body temperature.

The injection was done under ether anesthesia. The renal artery of the dog was exposed by retroperitoneal approach, dissected free from the pedicle, encircled by a thread and temporarily constricted by manual traction. The freshly prepared and warmed trypsin suspension was placed in a 10 cc. syringe to which a curved hypodermic needle was attached. The artery was entered and 8 to 10 cc. of the suspension slowly injected over a period of 2 to 3 minutes. The artery was kept constricted for 2 minutes more after the end of the injection. This slow injection and temporary renal artery occlusion was found to be essential in order to produce the renal damage. Preliminary experiments showed that faster injections and shorter artery occlusions were ineffective, apparently because the rapid blood flow diluted and washed away the trypsin. Further, it was found that the daily injection of 20 cc. of this trypsin suspension into the brachial vein of normal dogs over a period of several months did not damage the kidneys or cause the blood pressure to rise.

(b) *The Production of Renal Ischemia.*—Under ether anesthesia the renal artery was dissected free *via* retroperitoneal approach, a Goldblatt clamp applied, the clamp closed completely and then released one complete turn.

(c) *The Determination of the Blood Pressure.*—All blood pressures were determined by means of a calibrated Hamilton recording manometer (31) connected to a needle inserted directly into the femoral artery. The dogs with one exception (K-2) were trained for this procedure, and a basal control level was obtained in repeated daily readings before any operative procedures were done. No anesthesia was found necessary for the blood pressure determinations. The dog would show little or no reaction to the needle puncture. Details of the procedure are given in a previous communication (32).

(d) *The Blood Non-Protein Nitrogen Determination and Urinary Concentration Test.*—Blood non-protein nitrogens were determined in the usual manner using the Koch method (33).

Urinary concentration was determined after the dog had been deprived of water for 24 hours. At the end of this period the dog was watched until it urinated. This first urine was discarded and the second urine when collected was used for the specific gravity determinations. Water was withheld until this second urine was collected. The first urine was discarded because many dogs were found to retain urine in their bladders for over 24 hours, thus making this urine unsuitable for concentration measurements.

RESULTS

1. The Injection of Trypsin into Both Renal Arteries of the Normal Dog.—Thirteen dogs were used in this study, of which six died or were sacrificed within 48 hours, the remainder living for a period of from 1 to 12 weeks. In one, T-57, the injection was made in the remaining kidney, the other having been removed at the time of the

operation.   Table I is a summary of the results in these animals and Text-figs. 1 to 3 show the essential findings graphically.

(a) *Effect of Trypsin Damage on the Gross and Histological Appearance of the Kidneys.*—

Acute Changes.—All six animals examined within 48 hours after trypsin injection had kidneys which showed, on gross examination, marked edema and large areas of hemorrhage and necrosis of varying extent.   In those dogs dying naturally within this period, the entire kidney substance appeared to be necrotic.   On microscopic examination (Fig. 1) the picture was predominantly one of severe necrosis with widespread hemorrhage and polymorphonuclear leucocyte infiltration of the interstitial tissue, glomerular capsules and lumina of the tubules. Tubular autolysis and glomerular compression were also present.   The tubules appeared to be damaged the most and necrosis appeared chiefly here.   The arteries and arterioles, on the other hand, appeared to be uninvolved.   The damage histologically was patchy, and sharp boundaries were discernible between the damaged and the apparently normal kidney substance.

Chronic Changes.—The kidneys of the seven dogs dying or sacrificed 1 to 12 weeks after the trypsin injection showed a different picture from the above.   On gross examination, all the kidneys appeared to be greatly contracted, with large depressed yellow scars occupying as much as half of the kidney.   Between the scars, the kidney appeared normal in architecture and color.   On microscopic examination, there were focal areas of extensive fibrosis with complete obliteration of glomeruli and occasional dilatation of the tubules.   It is important to point out that there were no indications of a persisting inflammatory or other parenchymal change either in these scarred areas or elsewhere in the kidneys.   No indications were found in any of the kidneys of crescent formation or chronic vascular changes.   The contrast between the findings in the scarred area and the normal areas between scars is striking macroscopically, as a comparison of Figs. 2 and 3, both taken from the same kidney, will show.   In the latter area the glomeruli and tubules appear normal.   In short, the picture is one of a normal kidney with large areas of scar formation, indicating that the injection of trypsin had produced a focal acute involvement which had resolved into focal scarring.
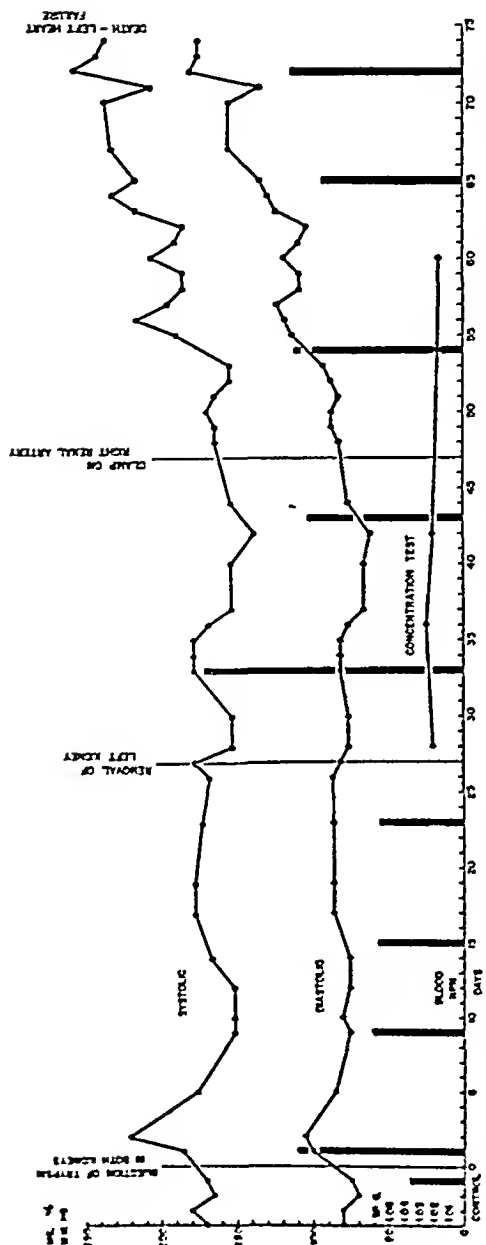
TEXT-FIG. 1. Effect of trypsin damage to the kidneys on the blood pressure, blood non-protein nitrogen and concentration ability of the kidney in K-1. Also the effect of removing one of the trypsin-damaged kidneys, and of the later production of ischemia in the remaining trypsin-damaged kidney.
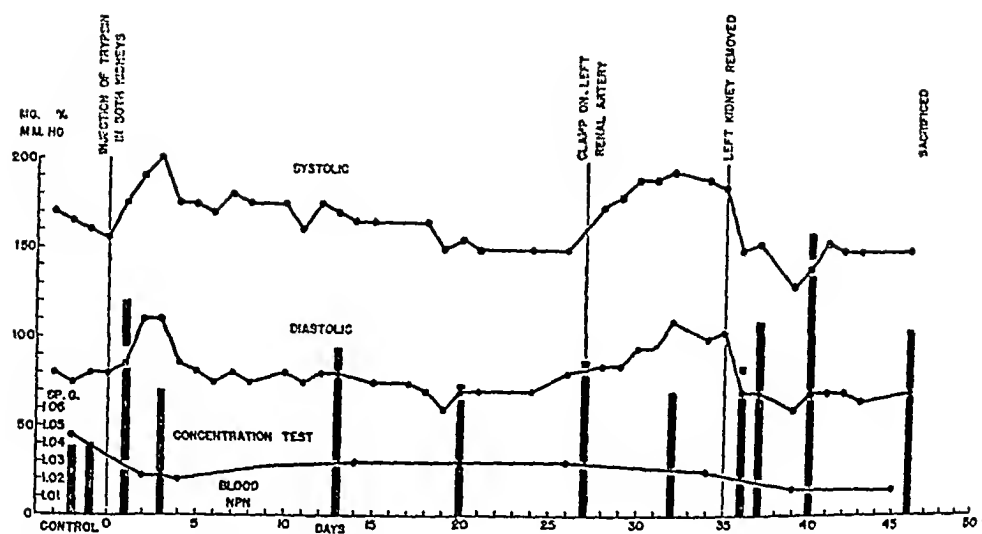
TEXT-FIG. 2. Effect of trypsin damage to the kidneys on the blood pressure, blood non-protein nitrogen and concentration ability of the kidney in K-9. Also the effect of renal ischemia of one of these trypsin-damaged kidneys and later removal of this ischemic and trypsin-damaged kidney.
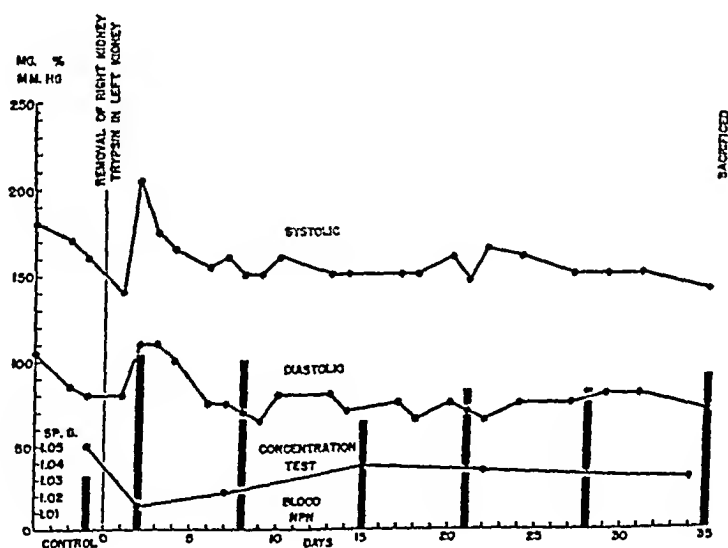
TEXT-FIG. 3. Effect of trypsin damage to the remaining kidney (the other having been removed) on the blood pressure, blood non-protein nitrogen and concentration ability of the kidney in T-57.

(b) *Effect of Trypsin Damage of the Kidneys on the Urinary Findings.* —For the first day or two, there was usually an anuria. The first urine voided always contained red blood cells, white blood cells, granular and hyaline casts and large amounts of albumin. As can be seen in Table I, the red blood cells in the urine tended to disappear within 2 weeks, but the casts and albumin could still be found for weeks. The most persistent abnormality was the albuminuria. In some dogs the albuminuria, slight in degree, lasted for the duration of the experiment.

(c) *Effect of Trypsin Damage of the Kidneys on Excretory Efficiency.* —A severe renal excretory insufficiency was produced in all dogs, as can be seen in Table I and Text-figs. 1 to 3. In every case, blood nitrogen retention occurred and lasted throughout the course of the experiment, but it was usually greatest during the first week. In one dog, K-1, removing one of the kidneys injected with trypsin 4 weeks after the injection, elevated the blood nitrogen for the following 3 weeks.

The concentration test revealed a great depression in the ability of the kidneys to concentrate the urine (*cf.* Table I and Text-figs. 1 to 3). The greatest depression occurred in the first 2 weeks, the specific gravity of the concentrated urine falling, in two dogs, to as low as 1.014 compared with the normal value of 1.040 and 1.050. However, after the first week, the specific gravity of the concentrated urine tended to rise, but in no instance did it return to the control level.

(d) *Effect of Trypsin Damage of the Kidneys upon Blood Pressure.*— Daily blood pressures revealed a postoperative hypertension lasting a few days (Table I and Text-figs. 1 to 3). The maximum pressure occurred usually on the 3rd day, the rise being on the average 35 mm. Hg for the systolic pressure and 30 mm. for the diastolic. By the end of the first week the blood pressure was found to be back to normal, or even slightly below normal, despite the continuation of the renal excretory insufficiency; this state of affairs lasted as long as 3 months.[:]

[:] The longer persistence of the high blood pressure in K-2 probably represents the effect of lack of training of this dog, since similar apparent blood pressure elevations have been found by us to be present in over 60 dogs before they were trained. The training of K-2 was not begun until after the trypsin was injected.

Effect of Trypsin Injection into Renal Art...

Dog	K-1						K-2*						K-9					
	Blood pressure	NPN	Concentration test	Urinary findings			Blood pressure	NPN	Concentration test	Urinary findings			Blood pressure	NPN	Concentration test	Urinary findings		
				Albumin	RBC	Casts				Albumin	RBC	Casts				Albumin	RBC	Casts
	mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.			
Control values	170/80	34						32	1.040	0	0	0	160/80	39	1.055	0	0	0
Type of experiment	Trypsin in both kidneys						Trypsin in both kidneys						Trypsin in both kidneys					
Days 1–7, 1		101		0	0	0		84		0	0	0	175/85	120		0	0	
2	185/105	110		3+	3+	3+		90		0	0	0	190/110		1.020	3+	3+	
3	220/105	80						69		3+	3+	3+	200/110	70	1.022	3+	3+	
4		69											175/85	64	1.020	3+	3+	
5													175/80	54		3+	3+	
6	175/85	67						92					170/75	54				
7								90					180/80	50				
Days 7–14†	150/75	60	1.018				218/105	88					170/80	94	1.030	3+	2+	
14–21	170/80	56					210/105	70	1.014				160/70	92	1.030	2+	0	
21–28	170/80	55					170/85	136		+	0	2+	150/70	88	1.030	0	0	
28–35	Left kidney removed						175/90	98					Left renal artery occlusion					
	170/80	164	1.020										185/85	68	1.025	0	0	+
35–42	160/70	100	1.020	+	0	0	160/80	98	1.022				Left kidney removed					
42–49	150/70	57	1.020	+	0	0	170/85	46		+	0	0	145/70	160	1.015	+	0	
	Right renal artery occlusion												150/70	105	1.015			
49–56	160/90	108	1.015				170/80	110	1.028	+	0	0						
56–63	190/110	96	1.018	0	0	0	165/80	66	1.030	+	0	0						
63–70	220/125	90					165/80	50		+	0	0						
70–77	230/155	110					Right renal artery occlusion											
77–84							205/115	88	1.020	+	0	0						
							205/105	150		0	0	0						
Cause of death	Left ventricular failure						Uremia						Sacrificed					
Necropsy findings	Both kidneys extensively scarred but portions of kidney between scars normal both in the gross and microscopically						Both kidneys extensively scarred with portions of kidney between scar areas normal both in the gross and microscopically. Kidneys were extremely shrunken						Left kidney very shrunken with large focal scars but normal tissue between these scar areas. Right kidney small, contracted with multiple small depressed scars					

* No control blood pressures taken on K-2.
† After first week, average blood pressure is given and non-protein values are maximum values in ...

...al Structure, Function and Blood Pressure

T-41				T-47						T-55						T-57					
Concentration test	Albumin	RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts
sp. gr.				mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.			
	0	0	0	155/80	38					160/85	50	1.050	0	0	0	170/90	30	1.050	0	0	0
in both kidneys				Trypsin in both kidneys						Trypsin in both kidneys						Trypsin into left kidney Removal of right kidney					
	3+	2+	3+	150/85	50		3+	2+	3+	110/60	75					140/80	74		0	0	0
				160/85	53					160/85		1.014	2+	3+	3+	205/110	104	1.014	3+	3+	3+
				185/110						175/95	60					175/110					
				155/80						160/95						165/100			3+	3+	3+
										170/95	82		2+	2+	2+	155/75	84				
				145/75	76					160/85	88					160/70		1.022	2+	0	2+
				140/75	54					160/85	88	1.018	2+	2+	2+	150/75	100	1.022	+	0	3+
				140/75	60					Left renal artery occlusion											
										185/105	150					140/75	68	1.038	+	0	+
										180/100	240	1.015	+	0	0	155/70	82	1.035			
																145/75	90	1.030	+	0	0

c peritonitis

Rupture of incision with peritonitis

Uremia

Sacrificed

showed exten-
with large
areas closely
huge infarcts
appearing
n infarcts

Both kidneys showed extensive focal damage with old hemorrhagic areas still present with great increase in connective tissue in these areas of former necrosis. Remainder of kidney normal

Left kidney showed a large hemorrhagic cyst almost as large as the kidney. Large scars throughout kidney with normal tissue between. Right kidney was the seat of an acute infection

Right kidney normal in every respect. Left kidney showed large depressed focal scars involving cortex and medulla with normal tissue between scars

Effect of Trypsin Injection into Renal ...

Dog	K-1						K-2*						K-9					
	Blood pressure	NPN	Concentration test	Urinary findings			Blood pressure	NPN	Concentration test	Urinary findings			Blood pressure	NPN	Concentration test	Urinary findings		
				Albumin	RBC	Casts				Albumin	RBC	Casts				Albumin	RBC	Casts
	mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.			
Control values	170/80	34						32	1.040	0	0	0	160/80	39	1.055	0	0	
Type of experiment	Trypsin in both kidneys						Trypsin in both kidneys						Trypsin in both kidneys					
Days 1–7, 1		101						84		0	0	0	175/85	120		0	0	
2	185/105	110		0	0	0		90		0	0	0	190/110		1.020	3+	3+	
3	220/105	80		3+	3+	3+		69		3+	3+	3+	200/110	70	1.022	3+	3+	
4		69											175/85	64	1.020	3+	3+	
5													175/80	54		3+	3+	
6	175/85	67						92					170/75	54				
7								90					180/80	50				
Days 7–14†	150/75	60	1.018				218/105	88					170/80	94	1.030	3+	2+	
14–21	170/80	56					210/105	70	1.014				160/70	92	1.030	2+	0	
21–28	170/80	55					170/85	136		+	0	2+	150/70	88	1.030	0	0	
	Left kidney removed												Left renal artery occlusion					
28–35	170/80	164	1.020				175/90	98					185/85	68	1.025	0	0	+
													Left kidney removed					
35–42	160/70	100	1.020	+	0	0	160/80	98	1.022									
42–49	150/70	57	1.020	+	0	0	170/85	46		+	0	0	145/70	160	1.015	+	0	0
	Right renal artery occlusion												150/70	105	1.015			
49–56	160/90	108	1.015				170/80	110	1.028	+	0	0						
56–63	190/110	96	1.018	0	0	0	165/80	66	1.030	+	0	0						
63–70	220/125	90					165/80	50		+	0	0						
							Right renal artery occlusion											
70–77	230/155	110					205/115	88	1.020	+	0	0						
77–84							205/105	150		0	0	0						
Cause of death	Left ventricular failure						Uremia						Sacrificed					
Necropsy findings	Both kidneys extensively scarred but portions of kidney between scars normal both in the gross and microscopically						Both kidneys extensively scarred with portions of kidney between scar areas normal both in the gross and microscopically. Kidneys were extremely shrunken						Left kidney very shrunken with large focal scars but normal tissue between these scar areas. Right kidney small, contracted with multiple small depressed scars					

* No control blood pressures taken on K-2.

† After first week, average blood pressure is given and non-protein values are maximum values in e...

re, Function and Blood Pressure

			T-47						T-55						T-57						
RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts	Blood pressure	NPN	Concentration test	Albumin	RBC	Casts		
		mm. Hg	*mg. per cent*	*sp. gr.*				*mm. Hg*	*mg. per cent*	*sp. gr.*				*mm. Hg*	*mg. per cent*	*sp. gr.*					
0	0	155/80	33					160/85	50	1.050	0	0	0	170/90	30	1.050	0	0	0		
neys		Trypsin in both kidneys						Trypsin in both kidneys						Trypsin into left kidney Removal of right kidney							
2+	3+	150/85	50		3+	2+	3+	110/60	75		0	0	0	140/80	74		0	0	0		
		160/85	53					160/85		1.014	2+	3+	3+	205/110	104	1.014	3+	3+	3+		
		185/110						175/95	60					175/110			3+	3+	3+		
								160/95			2+	2+	2+	165/100		✦					
		155/80	77						82					155/75	84						
		145/75	76					170/95						160/70		1.022	2+	0	2+		
		140/75	54						85					150/75	100	1.022	+	0	3+		
								160/85	88	1.018	2+	2+	2+								
								Left renal artery occlusion						140/75	68	1.038					
		140/75	60					185/105	150					155/70	82	1.035	+	0	+		
								180/100	240	1.015	+	0	0								
														145/75	90	1.030	+	0	0		

tonitis	Rupture of incision with peritonitis	Uremia	Sacrificed
ed exten- th large s closely infarcts appearing farcts	Both kidneys showed extensive focal damage with old hemorrhagic areas still present with great increase in connective tissue in these areas of former necrosis. Remainder of kidney normal	Left kidney showed a large hemorrhagic cyst almost as large as the kidney. Large scars throughout kidney with normal tissue between. Right kidney was the seat of an acute infection	Right kidney normal in every respect. Left kidney showed large depressed focal scars involving cortex and medulla with normal tissue between scars

(e) *Effect of Trypsin Damage of the Kidneys on General Condition of Dogs.*—Immediately after the trypsin injection, all the animals appeared acutely ill, with symptoms of lassitude, weakness and anorexia. After a few days, however, six of the seven dogs that survived appeared in good condition, ate well and were lively. Gradually, however, the animals that survived lost weight and developed an anemia. None of the animals revealed any evidence of edema. This loss of weight and progressive anemia was not observed in control animals not having trypsin renal insufficiency, and is, therefore, apparently caused by the renal damage.

2. *The Effect of Renal Ischemia in Dogs with Chronic Renal Excretory Insufficiency.*—Unilateral renal ischemia was produced by the application of a Goldblatt clamp in four dogs (K-1, K-2, K-9 and T-55) which had previously been subjected to bilateral renal artery injection of trypsin 2 to 10 weeks previously and which as a consequence had a chronic renal excretory insufficiency. One dog (K-1) had one of its kidneys removed 3 weeks prior to the application of the Goldblatt clamp. Table I and Text-figs. 1 and 2 show clearly that following the application of the clamp, these dogs developed a marked hypertension, the average rise being 45 mm. Hg for the systolic and 35 mm. Hg for the diastolic pressures. The elevation in blood pressure in all instances was higher than the early transitory rise during the first few days of the acute stage of trypsin renal damage. It was observed too that three of these unilaterally clamped dogs showed an exaggeration in the renal excretory insufficiency following the production of renal ischemia. This was so marked in two of them that death in uremia resulted. In one (K-1) (Table I and Text-fig. 1) the acute hypertension which developed was so great that it led to death from acute left heart failure.

The record of K-9 (Table I and Text-fig. 2) is significant. In this dog unilateral renal ischemia caused a rise in blood pressure without altering the renal excretory insufficiency to any extent. However, removal of this trypsin-damaged ischemic kidney caused a fall of blood pressure to the level existing before the clamping where it remained, although both the blood non-protein nitrogen and the concentration test showed an aggravation of the renal excretory insufficiency.

The complete differentiation between hypertension and renal excretory insufficiency in these four dogs indicates unequivocally for the first time that renal excretory insufficiency, *per se,* does not produce hypertension even when the animal can be shown to be potentially capable of developing hypertension. The rapid and marked elevation of blood pressure following the application of the Goldblatt clamp to one of these trypsin-damaged kidneys indicates further that these dogs with chronic renal excretory insufficiency were not too cachectic to develop and maintain a hypertension before the clamp was applied.
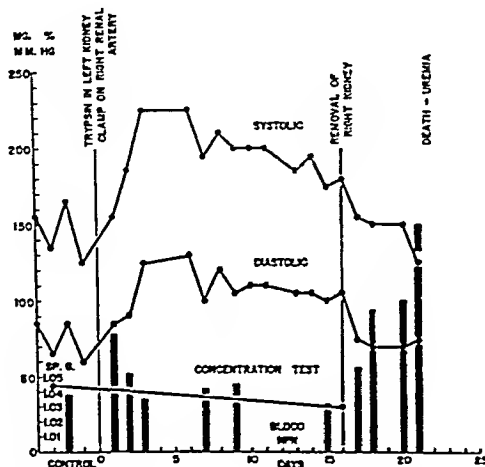
TEXT-FIG. 4. Effect of simultaneous trypsin damage to one kidney and production of ischemia in the other on the blood pressure, blood non-protein nitrogen and concentration ability of the kidney in T-59. Also the effect of later removal of the ischemic kidney.

It indicates further that the absence of hypertension was due to the absence of the mechanism that produced hypertension and not to the inability of the animal to respond to the renal excretory insufficiency with a hypertension.

3. *Effect of Combined Unilateral Renal Ischemia with Trypsin Damage to the Other Kidney.*—In a series of four dogs, unilateral renal ischemia by renal artery clamp was produced simultaneously with trypsin damage to the other kidney. The results are shown in Table II and Text-fig. 4. All four dogs developed a hypertension; in

Effect of Unilateral Renal Ischemia and Simultaneous ...

Dog..........	T-59						T-62			
	Blood pressure	NPN	Concentration test	Urinary findings			Blood pressure	NPN	Concentration test	Albumin
				Albumin	RBC	Casts				
	mm. Hg	mg. per cent	sp. gr.				mm. Hg	mg. per cent	sp. gr.	
Control values	130/70	37	1.046	0	0	0	150/80	34	1.050	0
Days 1	155/85	78	Operation						Operation	
2	185/90	52					155/95	116	1.027	3+
3	225/125									
4	180/95	34					180/110	76		
5	225/130			3+	3+	3+	155/100	82		
6							160/110	110		
7	190/100	42					160/100	74		
8	200/110									
9	200/105	45								
10	200/110									
11	200/110									
12										
13	185/105									
14	195/105									
15	175/100	32								
16	180/105		1.030	+	0	0				
			(Ischemic kidney removed)							
17	150/75	65								
18	150/70	94								
19										
20	150/70	100								
21	125/65	150								
Cause of death	Uremia						Distemper			
Necropsy findings	Left kidney extensively scarred and contracted with normal areas of tissue between scars. Right kidney normal with clamp on renal artery						Left kidney almost completely nec... hemorrhage throughout, and t... size of right kidney. Right kidn... with clamp on renal artery			

...ther Kidney on Renal Function and Blood Pressure

T-60

Blood pressure (mm. Hg)	NPN (mg. per cent)	Concentration test (sp. gr.)	Albumin	RBC	Casts
175/85	44	1.055	0	0	0
		Operation			
205/125	64	1.015	3+	3+	3+
220/125	118				
220/125	106				

Hemorrhage

Left kidney swollen to twice the size of right with large areas of hemorrhage and necrosis. Right kidney was normal with clamp on renal artery

T-64

Blood pressure (mm. Hg)	NPN (mg. per cent)	Concentration test (sp. gr.)	Albumin	RBC	Casts
150/80	34	1.050	0	0	0
		Operation			
165/95	44		2+	0	3+
175/100	45				
185/105	43	1.045	2+	0	3+
175/95					
175/105					
175/95	40				
200/125					
165/100					

Distemper

Left kidney edematous with large areas of granulation tissue and old hemorrhage. Right kidney normal in appearance with clamp on renal artery

two (T-62 and T-64) with distemper, this was not marked. In the other two, it was more intense. All the dogs showed evidence of renal structural damage in the trypsin-injected kidney at autopsy, and three showed signs of renal excretory insufficiency during life.

One of these dogs (T-59) was followed for 16 days and it was found that as the hypertension developed the blood non-protein nitrogen returned to its normal value. On the 16th day the ischemic kidney was removed, leaving behind only the trypsin-damaged kidney. This resulted in a fall in blood pressure to the control level, and at the same time, a rise in blood non-protein nitrogen indicating a progressive renal excretory insufficiency which led to the death of the animal in uremia. This experience again indicates the sharp differentiation between renal excretory insufficiency and hypertension and the absence of any direct relationship between the two. It shows that the ischemic kidney was responsible for the hypertension, although this might have been facilitated by the presence of renal excretory insufficiency in the other kidney. It also shows that the ischemic kidney retained sufficient excretory function to minimize the effect of the damage done by the trypsin in the other kidney.

DISCUSSION

Our experiments indicate that hypertension is not due to chronic renal excretory insufficiency even when this is of marked degree. The lack of hypertension in chronic renal excretory insufficiency cannot be attributed to the inability of these animals to develop hypertension as suggested by Pässler and Heineke (19) and Chanutin and Ferris (20) who pointed out that cachexia in itself can mask the action of the hypertensive mechanism. The fact that four of our dogs with chronic renal excretory insufficiency developed a definite hypertension following the application of a Goldblatt clamp to the renal artery of one of the trypsin-damaged kidneys, indicates that the dogs were not too weak to develop hypertension, had the mechanism for its production been present.

In evaluating the blood pressure changes in renal excretory insufficiency it is important that the method and manner of determining the

M. FRIEDMAN AND L. N. KATZ

blood pressure be accurate and relatively constant. The employment of various indirect methods, the use of anesthesia during a blood pressure determination and reliance on single determinations of blood pressure even in a large number of animals, as have been done in many previous experiments, introduce the possibility of widely divergent results. It is not impossible that the real cause of the confusion regarding the interrelation of renal excretory insufficiency and hypertension is on this basis. Our use of the Hamilton apparatus allowing direct measurements of the systolic and diastolic pressures in unanesthetized, trained dogs and the determination of the pressure daily for long periods of time, appear to us to reduce the possible error in blood pressure measurements to a negligible factor.

In most previous studies in which renal excretory insufficiency was produced, the possibility was not excluded that renal ischemia, which is known to cause hypertension, coexisted. Thus, several workers (23, 24, 26) reported vascular and glomerular damage in the kidneys having renal excretory insufficiency. Jarrett et al. (34) found that vascular damage was present after partial nephrectomy severe enough to produce renal excretory insufficiency, and Wood and Ethridge (21) found such vascular damage 6 months after partial nephrectomy, at the time that an elevated blood pressure occurred. In most of the procedures hitherto employed it has not been possible to limit the damage only to the elimination of excretory units in the kidneys, without the possibility of interfering simultaneously with the blood supply to the remaining kidney substance. Our previous work (29) has shown that the development of hypertension by ischemia is dependent upon the ratio of ischemic to normal kidney substance present rather than on the amount of ischemic kidney alone. It therefore follows that less ischemic kidney is needed to produce hypertension as the number of normal kidney excretory units is decreased. Slight degrees of renal ischemia without effect when the kidneys are normal prior to its induction, may on this account readily lead to hypertension when the number of normal excretory units of the kidney are reduced.

Our results are clear, we believe, in showing that renal excretory

insufficiency develops when the total number of effective excretory units in the kidneys are reduced beyond a minimal critical value. There is no need to have a continued insult or repeated insults to lead to this stage; a single insult applied only once is sufficient, if it causes extensive enough damage. Apparently a minimum number of effective excretory units is essential to avoid renal excretory insufficiency. This concept explains the lack of correlation between the presence of active parenchymal damage and renal excretory insufficiency. Immediately following the injection of trypsin into both kidneys, there occurs acute damage to the kidney parenchyma, acute renal excretory insufficiency and hypertension. At this stage, it would have been impossible to relate the hypertension to either the damaged parenchyma or the altered excretory function of the kidney. However, with the complete invasion of the localized necrotic areas of the kidney by scar tissue, leaving normal kidney tissue between the scar areas, it was seen that no subacute or chronically active process involving the kidney parenchyma was present. Coincident with this change in renal histological appearance, the blood pressure fell to normal and remained so although the renal excretory insufficiency continued. Thus it was discerned that renal excretory insufficiency persisted in the absence of any active continuing damage to the kidney.

The absence of hypertension in chronic renal excretory insufficiency indicates that the latter is not the mechanism responsible for renal hypertension, although it doubtless contributes to the ease of its production. Renal hypertension occurred in these experiments only when there was renal ischemia or active damage to the renal parenchyma. While the relation of hypertension to renal ischemia—clearly shown by the experiments with renal clamping and later removal of the ischemic kidney—accords with previous work on the subject, the relation of hypertension to active damage of renal parenchyma has not hitherto been demonstrated as far as we know. How the hypertension is produced during the period of renal damage immediately following trypsin injection is not established. Three possibilities exist: (a) the damage to the kidney may lead to a sustained pressor

reflex originating in the afferent nerves of the kidney; (b) the damage to the kidney by trypsin may liberate a substance which acts as a humoral mediator for the hypertension; (c) the swelling of the parenchyma of the kidney, resulting from the damage and operating within the restraint of the kidney capsule, may cause a compression of its blood supply with a consequent ischemia. Thus acute swelling of the kidney in the acute stage of trypsin damage would cause renal hypertension by ischemia of its substance in the same way as that produced by a renal arterial clamp.

SUMMARY

1. The injection of trypsin into both renal arteries of the dog was found to cause an acute necrosis of large sections of the kidney, an immediate excretory insufficiency, and a transient hypertension.

2. Dogs surviving the acute phase of the trypsin injection, developed a chronic renal excretory insufficiency with no hypertension, despite the severity and duration of the renal excretory insufficiency.

3. The application of a Goldblatt clamp to the renal artery of one of the two kidneys, previously injected with trypsin, led to a rise in blood pressure which returned at once to normal when the ischemic kidney was removed, even though the pre-existing renal excretory insufficiency was augmented. This experience demonstrated unequivocally that chronic renal excretory insufficiency and hypertension are not directly related.

4. The application of a Goldblatt clamp to the renal artery of one kidney and the simultaneous injection of trypsin into the other led to a hypertension. The later removal of the ischemic kidney led to a severe renal excretory insufficiency, at the same time the pre-existing hypertension disappeared. This indicated again that renal excretory insufficiency and renal ischemia produced different phenomena and that the former had no direct relation to hypertension.

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EXPLANATION OF PLATE 16

The sections were stained with hematoxylin and eosin.

FIG. 1. Acute effect of trypsin injection into the kidney. Section taken in B-1, 24 hours after the injection of trypsin. Widespread hemorrhage can be seen in the glomerular capsules, in the interstitial tissue and in the lumina of the tubules. Autolysis and desquamation of the tubuli and interstitial infiltration of polymorphonuclear leucocytes are clearly demonstrable. A glomerulus is shown, in which there is compression with infiltration of polymorphonuclear leucocytes and free red blood cells. × 140.

FIG. 2. Chronic change following trypsin injection into the kidney. Section taken in K-1, 4 weeks after the injection of trypsin. This section is from an area scarred in the gross. The complete obliteration of two glomeruli by scar replacement is shown. The increase in connective tissue, the infrequency of tubuli and the presence of round cell infiltration are clearly demonstrable. × 140.

FIG. 3. Chronic change following trypsin injection into the kidney. Section taken in K-1, 4 weeks after trypsin injection. This section is from a portion of the kidney appearing normal in the gross. The normal appearance of glomeruli and tubules is evident. The dark staining tubule is a photographic defect. × 160.

PLATE 16

STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

V. Sensitization to Diazomethane and Mustard Oil

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In continuation of studies on the production of hypersensitiveness to simple chemical compounds in animals, experiments have been carried out on sensitization to two non-aromatic substances, diazomethane and allylisothiocyanate.

While it would be rather pointless at present to extend anaphylactic experiments to a great variety of proteins which in general behave much alike, in spite of differences in their sensitizing activity (Doerr) and the apparent absence of this capacity in peculiar proteins such as gelatine, with simple chemical compounds, on the other hand, new problems are offered with each different group of substances as regards the possibility of inducing sensitization, the search for appropriate methods to attain this end, and the mode of action of the substances in the animal body. Indeed, it may be stated again, with a considerable number of substances which cause severe allergic disease in man experimental sensitization has not been surely achieved either in animals or in human beings, and this holds true even for cases where the formation of antigenic conjugates might be expected on chemical grounds. The two compounds investigated in the present article are readily capable of forming conjugates.

1. Diazomethane

Diazomethane is a yellow gas of the formula CH_2N_2. It is widely used in preparative chemistry on account of its high reactivity, especially for the introduction of methyl groups in acids, alcohols, and nitrogen compounds. Its toxicity was noticed by its discoverer von Pechmann (1) and the substance has since proved troublesome to laboratory workers. In part the effects were found to be attributable to a condition of hypersensitiveness as stated by Arndt (2).

According to this author, a person may be able to work with the compound for some time without untoward effects. Afterwards, however, hypersensitiveness may develop, even if precautions are taken, so that for such persons it is almost impossible to work with diazomethane without being subjected to severe attacks of asthma and fever. Experimental sensitization with the substance seems not to have been reported.

Our first attempts to sensitize guinea pigs were made with repeated applications of a solution of diazomethane in dioxane since ether solutions commonly used in chemical work evaporate too rapidly. The results were positive in part but were inconsistent. Several modifications of the procedure were tried and these experiments suggested that solvents having irritating properties in themselves give better results. In particular it seemed that dioxane containing peroxides such as is frequently met with was more suitable than pure dioxane. Positive results were also observed when cottonseed oil was employed as solvent.

The following method was finally found satisfactory in preliminary experiments and was then applied to a rather larger group of animals.

Diazomethane was prepared in the usual manner from nitrosomethylurethane but dioxane was substituted for ether as solvent and the gas was absorbed in cooled pure dioxane. The solution made in this way, containing 20 to 25 mg. CH_2N_2 per cc., was used in the tests. For sensitizing the solution was diluted with an equal volume of dioxane 0.01 molar with respect to organic peroxide (determined iodometrically), obtained by concentration *in vacuo* of commercial dioxane.

The solution of dioxane was applied to the skin of the haunch, the hair being removed before each application by means of an electric clipper. 10 drops were allowed to fall from a capillary pipette onto the skin and this was repeated twice at intervals of about 15 minutes, totalling 6 to 8 mg. diazomethane per day per animal. Obviously, a large part of the substance evaporated since the site became dry after a short time. As the treatment continued, the treated site became pink and rough and layers of scales developed. The skin beneath the scales remained unbroken in most cases. In order to secure contact of the solution with the skin the scales were removed when necessary by clipping after softening with olive oil. After the animals had received twelve such treatments within 2 weeks, they were tested 3 weeks later by applying a saturated solution of diazomethane in pure dioxane, as mentioned above, for 2 or 3 successive days, on the flank.

A lot of 40 male albino guinea pigs weighing between 330 and 450 gm. were treated in the manner described.   The 38 surviving animals were tested by applying diazomethane solutions on 3 successive days. Of ten control animals similarly treated three had a very faint pink color on the site of application 24 hours after the third treatment, the others were practically negative.   Of the experimental animals 60 per cent showed definite reactions of varying degrees.   The reactions consisted in erythema ranging from faint pink to pink color. The test sites often were somewhat elevated, and thickening could be detected upon pinching up a fold of skin.   The best reactors, 12 in number, were selected and subjected to a second course of 8 treatments on the opposite haunch, followed 3 weeks later by test applications given on the unused flank on 2 successive days (controls 23 to 32).   The reactions observed in these animals after the first and the second course are tabulated (Table I).   From the table it appears that a second course of applications had distinctly increased the degree of sensitization.   When treatment of the sensitized animals was continued on one site, employing diazomethane without peroxide, the marked thickening and scaling of the skin appeared much more quickly than in non-sensitized animals even when in the latter there was the added effect of peroxides.

In the advanced stage microscopical examination showed that the epithelial layer was very much thickened (up to five times its normal depth), in proliferation, with increased keratin layer and papilli extending into the dermis.

A point of interest in the reported experiments concerns the nature of the sensitizing substance which is so highly reactive that it doubtless combines with substances of the animal body rapidly after administration and for this reason the spread of sensitization can hardly be ascribed to the distribution of the exciting substance itself but to transportation of some sort of conjugate[1] or, perhaps, of antibodies.

Although the profound change in the serological properties of proteins through methylation has been established in previous work (3), it is still undecided whether the sensitization effects described

[1] For the antigenic activity of methylated proteins, see Landsteiner (3).

TABLE I

Reactions of animals sensitized to diazomethane, after one and two courses of treatments, and of non-sensitized control animals. The tests were made on a fresh site of the skin by applying a diazomethane solution as described in the text on each of 3 (or 2) successive days. Readings were made the day following each application.

No.	Reactions after first course			Reactions after second course	
	First application	Second application	Third application	First application	Second application
1	pp., mac.	pp.–p.	pp.–p., th., sc.	pp.	dp., th.
2	vfp.	fp.–pp., mac.	pp.–p., sl.sc.	pp., mac.	p.
3	pp., mac.	pp.	fp.–pp., sc.	pp.–p.	pp.
4	fp.–pp., mac.	p., sl.th.	pp.–p., th., sc.	p.	dp., sl.th.
5	vfp.	pp.	p., sc.	pp.	p., sl.th.
6	pp., mac.	p., sl.th.	p., sc.	bp., sl.th.	dp., th.
7	fp., mac.	pp.–p.	fp.–pp., sc.	pp.–p.	pp.–p.
8	pp.	p., sl.th.	pp.–p., sc.	bp., sl.th.	bp., th.
9	fp.–pp., mac.	pp.–p.	p., sc.	p.	no application
10	fp.	p.	pp.–p., sc.	bp., th.	bp., th.
11	vfp., mac.	pp.–p.	pp., sl.sc.	pp.–p.	p., sl.th.
12	fp., mac.	pp.	p., sl.th.	p.	dp.

Controls

13	neg.	neg.	neg.	23	neg.	fp., mac.
14	neg.	neg.	neg.	24	neg.	vfp., mac.
15	neg.	vfp.	neg.	25	vfp., mac.	fp.
16	neg.	neg.	neg.	26	fp., mac.	fp.–pp.
17	neg.	vfp.	neg.	27	neg.	al.neg.
18	neg.	al.neg.	al.neg.	28	neg.	fp.
19	neg.	al.neg.	al.neg.	29	vfp., mac.	vfp.
20	neg.	vfp.	al.neg.	30	fp., mac.	fp., mac.
21	neg.	neg.	neg.	31	al.neg.	vfp., mac.
22	vfp.	al.neg.	vfp.	32	neg.	al.neg.

The following abbreviations are used: negative (neg.), almost negative (al. neg.), faint pink (fp.), very faint pink (vfp.), pale pink (pp.), pink (p.), bright pink (bp.), dark pink (dp.), slightly thickened (sl.th.), thickened (th.), macular or spotted (mac.), slight scaling (sl.sc.), scaling (sc.).

are simply due to the formation of antigenic methyl proteins. In some cases unquestionable though slight anaphylactic reactions were seen in guinea pigs sensitized to diazomethane upon injection of

methylated guinea pig proteins.[2] This phase ought to be studied more extensively. Also, it has not yet been investigated whether skin sensitivity to diazomethane can be produced by injection of methylated proteins. In the study of some other substances an effect of this sort was not obtained (4).

Skin tests with other methylating chemicals gave definite cross reactions in the case of nitrosomethylurethane.[3] In a few cases methylsulfate produced intense skin reactions in sensitive animals but the results on reapplication were irregular and therefore are only mentioned incidentally.

The high toxicity of dimethylsulfate which at times has caused deaths in factory workers is in part due to its local corrosive action, in part to a systemic action. General toxic effects (6) have likewise been observed with methylchloride which has been widely used as refrigerant. In the literature we found only one casual remark (7) to the effect that individual idiosyncrasy may play a part in the poisoning by methylchloride. From the preceding, however, some attention, in our opinion, ought to be paid to the possibility of sensitization by methylating substances other than diazomethane.

2. Allylisothiocyanate (Mustard Oil)

Hypersensitiveness to mustard oil has been reported in two cases, to our knowledge, a small number in view of its not uncommon use as "counterirritant." Lehner and Rajka (8) described increased reactivity of the skin to mustard oil in a patient who had received eleven daily rubbings on the same site. The authors remarked that the individual appeared to be more sensitive than normal persons from the beginning. In a second similarly treated case Tezner (9) claims that he obtained local, not general, sensitization of the skin, showing immediate but no delayed skin reactions.[4]

On account of these reports it was deemed of interest to investigate the possibility of sensitizing animals and also to repeat the test with human beings.

[2] It should be mentioned that in these animals some superficial sores had developed on the treated site.

[3] On the use of this compound and of nitrosomethylurea as methylating agents, see (5).

[4] It may be mentioned that an increase in resistance to mustard oil on repeated administration to the skin of rabbits has been reported by Saudek (10).

Six persons from our laboratory were treated on 6 days each week for 3 weeks by allowing 1 drop of synthetic mustard oil to fall onto the skin of the forearm. This was followed by immediate hyperemia which faded soon. In five of these individuals there was no significant change except for a few slight transient reactions (delayed) in two individuals which may perhaps indicate a very low grade of sensitization. The sixth person, however, developed distinct hypersensitiveness. In this case, on the 13th application on the same site, an erythematous reaction began to appear after about 12 hours, and on the following day the site was intensely red, sharply demarcated, and slightly elevated, whereas before in this and the other individuals no reaction, or only a faint color was to be seen on the next day. The erythema began to fade on the 3rd day and gradually the site became brownish. When the sudden increase in reactivity was first observed, two other sites on the arms were tested and reactions developed similar to that described. On the next day, several other areas were tested (chest, back, both legs, and one arm) with positive results. Only minor differences in the intensity of the reaction were seen in the various regions. On testing fresh sites on the arm with drops of various dilutions of mustard oil in absolute alcohol, a definite though not intense reaction was still seen with a dilution of 1:20.

In order possibly to increase the sensitivity another course of 15 applications was given this individual 10 weeks later. Almost 3 weeks after the termination of the second course, the skin was still definitely hypersensitive, but the intensity of the reactions was somewhat diminished; a month later a new test site showed again a very distinct reaction.

Attempts to sensitize animals were made with guinea pigs by repeated superficial application and also by intracutaneous injection of mustard oil diluted with olive oil. In this species, in three monkeys, and three rabbits no definitely positive results were obtained. On the other hand, of three young hogs (Chester Whites) treated in similar manner as the human beings, two became distinctly hypersensitive.

One animal which had received eighteen superficial treatments on two sites, within 3 weeks, was tested on a new site by gently spreading 1 drop of mustard oil with a glass rod. It gave a pronounced reaction, whereas before sensitization this animal and also other normal pigs

showed no or very light erythema, only exceptionally a somewhat stronger reaction, on the day following an application of mustard oil. A second course seemed to increase the sensitivity.

Another male hog, after having received eight applications of a drop of mustard oil within 10 days on the same site, showed a strong reaction—pink elevated area seen on the following day—when tested on a new area with a drop of the substance. Further treatment of the animal resulted in some increase of sensitivity. A "titration" gave positive reactions with as little as a drop of a 5 per cent solution in dioxane.

In all instances, in contrast to the statement of Tezner, the skin sensitivity was not local but general, and the reactions were delayed, not immediate. In some of our tests the erythema at once following upon application of the substance seemed to be somewhat more pronounced and more lasting in sensitive than in non-sensitive individuals, but the differences were too small to be considered as significant. Furthermore, experiments made in view of the claims by Lehner and Rajka—of importance if correct—that they were able to demonstrate passive transfer of sensitivity with human serum in guinea pigs, gave negative results. When their procedure was used, normal animals also exhibited toxic symptoms not weaker than those in the "passively sensitized" ones.

As to the mode of sensitization with mustard oil, it may be pointed out that the substance reacts with amines and amino acids (11). Also, when we treated protein with mustard oil a reaction took place similar to that observed by Hopkins and Wormall (12) who were able to couple proteins with phenylisocyanate. Naturally, this does no permit one to decide whether a combination of isothiocyanate with protein or perhaps with some other substance in the body is responsib for the sensitization.

From the results described it would seem that swine are more a to develop hypersensitiveness to mustard oil than guinea pigs whi however, can be readily sensitized to a number of other chemica This may indicate species differences in the reaction to various s sitizing simple compounds. Whether in general hogs offer an vantage in similar studies with other substances remains to seen. Regarding the test with human beings it may be pointed

that only one out of six became definitely sensitive; hence there appears to exist considerable individual variation in the ability of man to become hypersensitive to mustard oil, and therefore this substance would probably be of use in the study of hereditary disposition to drug hypersensitiveness.

SUMMARY

With the view of making new types of chemicals accessible for investigations on drug hypersensitiveness, methods have been devised for sensitizing animals with diazomethane and mustard oil, two non-aromatic compounds.

Guinea pigs have been sensitized to diazomethane, a substance of high reactivity and known to cause severe allergic effects in man.

With the second substance, allylisothiocyanate, likewise capable of forming conjugates with substances in the animal body, sensitization effects have been obtained in man and in hogs. Sensitization in human beings was successful with one out of six individuals treated.

The observations indicate species and individual differences as regards the ability to become sensitized to various chemical compounds.

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A PYOGENIC FILTERABLE AGENT IN THE ALBINO RAT

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PLATE 17

This paper describes, more fully than our preliminary communication (1), a filterable agent resembling the viruses which produces extensive necrosis and suppuration in certain tissues of the white rat.

One of us (2) has been carrying on for some years a search for antibodies against sarcoma 39 in rats which had cured themselves of this neoplasm, mixing emulsions of the tumor before inoculation with various organs of the rat or with adsorbents employed to remove an antibody from tissue extracts, or incubating them in such extracts or in Locke-Ringer solution. Though all materials had been proved sterile before use by ordinary bacteriological methods, large abscesses sometimes developed within a few days at the site where these tumor emulsions had been injected. This complication, which arose about twenty times in some 100 instances at irregular intervals throughout the course of five or six years, was at first ascribed to bacterial contamination, for which the requisite manipulations gave abundant opportunity. Aerobic and anaerobic cultures of the pus, however, were repeatedly negative in plain broth, dextrose broth, streptococcus broth, Rosenow's brain broth, Loeffler's blood serum, Huntoon's hormone medium, plain agar, blood agar, dextrose agar slants, Petroff's egg medium, blood plates in 10 per cent carbon dioxide, and the Bordet-Gengou medium. Organisms could not be found in smears stained after the methods of Gram, Wright, Ziehl-Neelsen, or with polychrome methylene blue, and, finally, no spirochetes were discovered upon dark field examination.

This consistent failure could not but suggest the participation of something resembling a virus, yet a search of the literature revealed only one virus indigenous to the rat,—reported by Novy (3) and quite different from the one now under discussion; and none in any animal species with activities so eminently pyogenic.

The Lesions in the White Rat

We have employed as standard material a 10 per cent tissue extract, made by grinding pus and abscess wall with sand in the appropriate amount of Locke-

Ringer solution without glucose, and centrifuging at moderate speed for 5 minutes. The customary dose has been 0.05 cc. of the supernatant fluid.

Subcutaneous Injection.—About 24 hours after inoculation there appears a slight puffiness which can be seen but not yet felt, and if the hair be thin some slight reddening may be discerned. On the 2nd day there is a palpable swelling, and by the 3rd its inflammatory nature is clearly apparent. The skin is hyperemic, the underlying tissue thickened, the outline diffuse, and the lesion about 2 cm. in diameter. At this time, though generally a few days later, the abscess may open and discharge its contents on the surface, an event which bears no relation to its size for smaller ones may evacuate spontaneously in this manner while larger ones may never do so. Many of the lesions, in fact, are absorbed under an unbroken skin during the course of several weeks.

In rats autopsied 24 hours after inoculation the subcutaneous tissues about the site are found to be slightly thickened and intensely congested, while through them runs a long whitish streak as though they had been seared by a hot wire, representing, no doubt, necrosis along the needle tract. On the 2nd day conditions are about the same except that this streak is now broader. On the 3rd the connective tissue is much more definitely hyperplastic, having somewhat the appearance and consistency of a rather soft fibroma, but in most cases free pus has not yet appeared. At this stage of the process the regional lymph nodes may begin to enlarge.

By the end of the first 24 hours the congestion has reached its height, for it is no more intense on the 2nd or 3rd day than on the first. On the 4th day (Fig. 1) free pus may or may not be seen but by the 5th or 6th it is uniformly present. An abscess removed at this time with its wall will weigh from 0.7 to 1.5 gm., about one-third of which will be accounted for by thick greenish yellow pus. By the 10th day the proliferative process is regressing and the abscess has become a thin walled pus sac (Fig. 2).

The amount of extract administered is immaterial within reasonable limits, doses of from 0.025 to 0.80 cc. having all produced large characteristic lesions. Even the highest of these doses caused no evident constitutional symptoms.

After the usual 0.05 cc. the animals move about normally, eat well, retain a glossy coat, gain weight, and none die. There does take place, however, a definite increase in the proportion of circulating polymorphonuclear leucocytes on the day after injection from the 20 per cent or so found in health to some 60 per cent, with a gradual return to normal during the next 7 days. The lymphocyte count, meanwhile, tends in the opposite direction. However, because of the widely recognized difficulty in obtaining reliable blood counts for the rat and the mouse, it is safer not to attach too much significance to these results for the present.

To preclude any chance that we were dealing with some sort of irritative lesion elicited merely by the products of tissue degeneration,

sterile abscesses were produced in rats by the subcutaneous adminis-
tration of turpentine, removed after intervals of 7 or 10 days, and
inoculated in the form either of tissue fragments or of 10 per cent
extracts. Among 9 such lesions, injected into 18 rats, not a single
one induced a response comparable in any way to that set up by the
agent. Similarly, the introduction of agent which had been inacti-
vated by heat produced no macroscopic changes.

Intravenous Injection.—Introduction of this filterable agent into the
blood stream in the form of tissue extract may be attended by conse-
quences far more serious than those following its subcutaneous
administration.

Seven groups of rats containing 42 animals in all were injected by way of a
caudal vein with 0.5 cc. of extract. They remained in apparent good health
for the next 2 or 3 days, but on the 4th redness and swelling of one or more paws
began to appear in most of the group, a characteristic lesion which is illustrated
in Fig. 3. By the 7th day, or thereabout, some 20 per cent of the animals were
moving themselves around by the forelegs, with paralyzed hindlegs trailing behind
in the supine position, a condition of which the cause has not yet been determined.

Within a day or so after injection there often set in an emaciation which
progressed so rapidly that about one-third of the body weight was lost in 1 or 2
weeks, though how much of the decrease was due to the infection itself and
how much to fasting entailed by difficulty in getting at the food supply cannot be
decided at present. That the latter may play an important rôle is suggested by
the empty gastrointestinal tract often encountered at autopsy, a condition which
cannot be referred to distaste for food because the animals ate eagerly when fed
from the hand.

In addition to these abscesses in the feet, collections of pus were found in the
testis, about the head, or in the soft parts surrounding the large joints, a more
or less characteristic site being the subcutaneous tissues cephalad to the scapula.
This localization, curious because no lymph node occupies this locality in the
rat and no connection with the hibernating gland could be demonstrated, occurred
in 5 animals of the intravenous group and in 2 out of 20 inoculated in the testis,
or in 7 among a total of 62, an incidence too high to be explained away by pure
chance. Furthermore, 3 rats had such an abscess at identical points on the
two sides.

In a few instances intravenous inoculation caused no apparent disturbance
of health; or, again, a rat that seemed hopelessly ill would eventually recover.
The mortality for the group as a whole was about 25 per cent.

The rectal temperatures in one lot of 12 animals, taken 3 days after inocula-
tion, were found to range from 98.8° to 101.8°, being above 100° in 9 instances,
while 3 days later none had a temperature higher than 100.6° despite the presence

of abscesses in the paws or about the large joints.   In 12 normal rats investigated at the same time a range of 97.6–98.8° was found.

Intratesticular Inoculation.—The testis is a vulnerable site, injection of a 1:1,000 dilution of the extract resulting in its total destruction by suppuration.

The introduction of 0.2 to 0.4 cc. of 10 per cent extract into a testis in 21 rats induced redness and swelling with eventual suppuration, the process becoming apparent about 2 days after inoculation and terminating in recovery by evacuation or absorption of the abscess during the following few weeks.   As in the case of intravenous inoculation, abscesses developed in the feet, in the soft tissues about a large joint, or at various points in the subcutaneous tissues, including the region cephalad to the scapula.

Intraperitoneal Inoculation.—The introduction of filterable agent by this route causes no apparent constitutional disturbance.

Of 12 rats thus injected with 0.5 cc. of extract none died.   Those killed for examination from 4 to 15 days later had abscesses along the needle tract, and in 3 instances the testis on the inoculated side was involved, probably by extension of the suppurative process.   Occasionally an abscess was discovered in the gastro-splenic or the great omentum, but never was there any generalized peritonitis. In 1 animal a small collection of pus was found in the subcutaneous tissues at the root of an ear, showing that the agent can affect a distant site after intraperitoneal injection, though it does not appear to do so as a rule.

Intracerebral Inoculation.—The results of intracerebral injection resemble somewhat those following inoculation by way of the blood stream.

Of 12 rats that had filterable agent introduced into the brain 3 developed the characteristic red and swollen feet and 1 of these the paralysis.   By the 4th day all had abscesses at the inoculation site in the subcutaneous tissues overlying the frontal bone, and in 3 that were killed for autopsy 18 and 22 days after inoculation an intracranial abscess was discovered which had evidently arisen by extension of the superficial lesion along the needle tract.   One of these had been sacrificed because it was found biting at a swollen hind foot, its behavior recalling in certain measure the reaction of animals to the intense itching of infectious bulbar paralysis, as described by Hurst (4).

Four of the rats suffered no apparent effect from the inoculation save for the subcutaneous abscess in the frontal region, 4 died and were not autopsied, and 1 with inflamed feet recovered.

WILLIAM H. WOGLOM AND JOEL WARREN

Percutaneous Inoculation.—The epidermis is exempt, a fact which may explain our failure to observe spontaneous transfer of the disease from infected to healthy rats.

Two drops of extract were rubbed thoroughly with a glass rod into the shaved and scarified skin of 6 rats. The abrasions were healing, or entirely healed, 4 days later with no sign of suppuration.

Distribution of the Filterable Agent

As the heart's blood elicited the typical abscess when withdrawn 7, 11, or 14 days after intravenous or intratesticular administration and subcutaneously injected into other rats, a wide distribution of the agent was only to be expected under these circumstances. Inoculation of various organs from rats infected by either of these two routes confirmed this anticipation for, with the exception of the suprarenal gland, the filterable agent was discovered at one time or another in all those tested; namely, the spleen, lumbar nodes, kidney, liver, and brain. It was not found, however, in the urine. On the other hand, 32 days after intravenous injection it had disappeared from the blood, lumbar nodes, liver, and kidney, only spleen and brain now producing suppurative lesions.

A more precise way of determining its tropism was to test the organs from rats with subcutaneous abscesses.

From the 1st to the 5th week following subcutaneous injection various organs were removed from 45 rats, belonging to different passages, and introduced under the skin in 685 others to see which would elicit the characteristic abscess. Where size permitted, as in the case of the spleen, the organ was reduced to a paste and injected in amounts of 0.10 cc., while smaller structures such as the suprarenal glands were halved and inoculated with a hollow needle. As experience had shown that sodium citrate did not interfere with the activities of the agent the heart's blood was drawn into a syringe containing a small crystal of this salt; here the dose ran from 0.4 to 1.5 cc. according to the amount available. The number of animals inoculated with each sample was dictated by its quantity. Emulsions of the larger organs sufficed for 6 rats, grafts of the lymph nodes for from 2 to 4, and of the suprarenal glands for 4.

The right axillary nodes were selected for testing because they were on the same side as the lesion yet not, like the lumbar group, imbedded in it, and the left axillary and the lumbar nodes because they represented distant ones.

As Text-fig. 1 shows, the agent has a distinct predilection for the lymph nodes and the brain, not having been found elsewhere save occasionally in the spleen and suprarenal gland. It is particularly worthy of note that although relatively enormous quantities must have been present at the site of the abscess, and some must have been distributed to the rest of the body, as attested by its recovery from distant organs, not once could it be demonstrated in sarcoma 39, though 17 neoplasms were examined from 4 to 34 days after their bearers had received a subcutaneous injection of filterable agent on the opposite side. The idea promptly suggested itself that this failure might indicate neutralization of the filterable agent within the sarcoma rather than failure to localize in the neoplasm, but it was abandoned for the time being when a tumor emulsion purposely incubated with agent was found to have caused no inactivation.

It should be pointed out that filterable agent was absent from, more often than present in, the organs tested; thus, for example, it was discovered in only 2 out of 23 spleens. These negative results constituted an automatic control, and obviated the necessity of examining organs from normal rats in order to preclude the possibility of a generalized infection among the animals of our strain.

Pathology

Tissues destined for microscopic examination were fixed in Zenker's fluid and stained with hematoxylin-eosin or with eosin-methylene blue.

Subcutaneously injected, the filterable agent causes widespread necrosis within 24 hours, soon followed by a monocytic reaction, permeation by polymorphonuclear leucocytes, and, after 4 or 5 days, the formation of a large densely encapsulated abscess. In the corium these changes are accelerated, and discrete pus may be found as early as the 1st or 2nd day (Fig. 4). We refer to the filterable agent as pyogenic because this suppurative lesion is its salient effect in the gross.

As for the internal organs, the only constant microscopic alteration was a lively reticulum cell reaction affecting the regional lymph nodes in the case of subcutaneous inoculation, or more generally distributed after intravenous administration. Throughout the remainder of the body, even when filterable agent had been introduced into the circulation, no histological changes were discovered which could not be

TEXT-FIG. 1. Distribution of the filterable agent after subcutaneous inoculation.

■, agent present. □, agent not found. ⊠, material not examined.

duplicated in presumably normal rats taken from stock. In particular, no perivascular infiltration was seen in the brain, no matter what the site of inoculation.

Nerves in the vicinity of subcutaneous abscesses showed no visible damage in sections stained with hematoxylin and eosin.

The suppurative lesions in the feet involved the soft parts only, the bone itself being spared though the marrow was thickly strewn with polymorphonuclear leucocytes.

Inclusion Bodies.—In the epithelium overlying abscesses of from 12 to 24 hours' duration a small number of eosinophile cytoplasmic structures have been found (Figs. 5 and 6) which closely resemble Guarnieri bodies, but Giemsa's and Goodpasture's stains, hematoxylin-eosin, and eosin, rosanilin, or phloxin in combination with methylene blue all failed to disclose inclusion bodies in sections of the various internal organs, of subcutaneous or intradermal abscesses, or of chick chorio-allantoic membrane or rabbit cornea inoculated with the agent. Though search has been made at intervals of from 2 hours to many days after inoculation it may be that the most propitious time has yet to be found.

Susceptibility of Other Species

Mouse.—This animal is particularly susceptible.

Twelve mice were inoculated subcutaneously, each with 0.05 cc. of extract, with the result that from 2 to 4 days later 9 had massive edematous swellings involving the whole side. Unlike rats injected in the same way these animals looked acutely ill, and most of them died within a week. In 2 that were killed for autopsy on the 3rd day the subcutaneous tissues were found hemorrhagic and necrotic, though without free pus, while microscopic examination revealed extensive permeation by polymorphonuclear leucocytes and a widespread necrosis. One animal lived until the 15th day, when it was sacrificed because it was moribund with an extensive abscess at the inoculation site.

Intracerebral injection in the mouse is followed almost invariably, on the 3rd day at the latest, by extensive edema of the head with suppuration in the subcutaneous tissues overlying the frontal region, where the needle entered the skull, and death generally ensues after from 4 to 11 days. Microscopic examination of the brain has revealed neither suppuration nor perivascular infiltration, the visible damage having been limited to a slight meningitis.

The filterable agent has been transmitted for 6 passages in the mouse brain, apparently with some loss of virulence for the rat at the end of the series.

Mice infected by nasal insufflation usually died of septic pneumonia after from to 17 days, only 3 out of 12 having escaped. The brains from 2 of these survivors, inoculated subcutaneously into rats and mice, elicited no abscesses; hence, ı all probability, filterable agent had not reached the brain as a consequence of he insufflation.

Guinea Pig.—Unlike the mouse, this species is highly refractory.

Following the subcutaneous introduction of 0.4 cc. of extract minute nodules, . few millimeters in diameter, appeared in 2 of the injected animals, while the ıther 2 developed no macroscopic reaction at the inoculation site.

Rabbit.—Subcutaneous injection is somewhat more successful than n the guinea pig.

Thus the administration of 0.5 cc. of extract produced slightly red flat tumefactions in all 4 rabbits inoculated by this route. These lesions were beginning to regress by the 4th day and were entirely healed after about 2 weeks. In 1 animal, killed for examination on the 6th day, the subcutaneous tissues appeared to be the seat of suppuration, though no free pus was found. The microscope revealed extensive leucocytic permeation.

A similar dose, introduced into the blood stream in 2 rabbits, caused no appreciable disturbance, while intracerebral inoculation with 0.4 cc. appeared equally harmless in the 1 rabbit thus injected.

Dropped upon the scarified cornea in 2 rabbits, the extract produced only conjunctivitis, which began to clear up within a week.

Chick Embryo.—A mouse strain has been maintained on the chorioallantoic membrane of the hen's egg, now for 10 generations, and is uniformly fatal for the mouse upon intracerebral inoculation. The presence of demonstrable agent may or may not be associated with the development of pin-point non-suppurative opacities on the membranes.

Immunity

Immunity, which is fairly well established by the 7th day after inoculation of the filterable agent, lasts for about 3 months, though it begins to diminish after 2.

Fifty-five rats that had recovered from intradermal, subcutaneous, intraperitoneal, or intratesticular inoculations were given a second injection from 7 to 101 days after the first one, the testing dose being administered subcutaneously and, in the case of those animals that had already received a subcutaneous or an

intradermal inoculation, on the opposite side of the body. About half the group proved to be entirely refractory and the remainder more or less so, for the resulting abscesses had a diameter exceeding 0.5 cm. in only 8; in 4 of these they attained the size of lesions in previously uninoculated rats.

This immunity was not accompanied by demonstrable neutralizing factors in the blood in 3 instances where signs of its presence were sought.

Serum from rats with 13 day old abscesses was combined with equal parts of a 1:100, 1:1,000, or 1:10,000 dilution of a 10 per cent saline extract, a similar series being set up with normal serum. After 90 minutes' incubation at 37°C. these mixtures were inoculated intratesticularly, together with appropriate controls of serum or undiluted extract alone. When the animals were examined 5 days later there was no definite evidence of neutralization.

A second test, with 11 day and 24 day citrated plasma, and a third with 8 day serum, were just as unsatisfactory, though in one of these the mixtures were kept in the ice box overnight following their incubation, and the testing inoculation was made at a different site, namely, in the subcutaneous tissues.

A macroscopic test for precipitins in 17 day and 24 day citrated plasma was negative.

An 0.5 per cent phenolized vaccine did not confer immunity in 5 rats treated with it.

Properties of the Filterable Agent

Filterability.—The agent can be passed through a Berkefeld N filter, though with considerable difficulty. In the preliminary account we said that it would traverse a W candle, but we have been unable to confirm this first result. The filter, used in connection with a water-tap aspirator of the common type, was impervious to the meningococcus both before and after the agent had passed through it, and the filtrate produced abscesses in 4 out of the 6 rats inoculated with it, but 4 subsequent attempts with a new W candle were entirely unsuccessful. Indeed, the agent will not always pass a Berkefeld N.

The attempts to filter through an N candle were made with lesions of different ages, extracted either in Locke-Ringer or in physiological saline solution. As a test of the candle, cultures of *Staphylococcus pyogenes albus* in plain broth were employed. In some experiments the culture was added at the beginning of the filtration; in others, when filtration had been half completed in order that the effect of the acid culture might be observed; while in still others plain broth was

added when filtration was started, or when it was half over, the test culture being employed at the end of the process. None of these variations made any difference in the outcome.

In 2 experiments the agent did not pass the N candle at all, for the filtrate elicited no lesions in 18 rats and 3 mice subcutaneously inoculated or in 3 mice intracerebrally injected. In 3 other trials the filtrate produced small abscesses in 9 out of 30 rats to which it was administered subcutaneously and in all 3 that received an intratesticular inoculation, or in 12 out of 33 altogether.

Infectivity.—Titration showed that a 10 per cent extract could be diluted to 10^{-2} without loss of infectivity. At 10^{-3}, 10^{-4}, and 10^{-5} some individual resistance began to appear in the injected rats, for in each of the 3 groups 1 animal was negative while the remaining 6, 2 in each lot, had small though characteristic lesions. At 10^{-6} and 10^{-7} no abscesses were produced.

Viability.—The filterable agent is attenuated by 30 or 60 minutes' exposure to 56°C., for extracts so treated did not elicit quite so many or quite such large abscesses. It is killed by heating for 1 hour at 60°C.

To ultraviolet light it is moderately susceptible. With a Cooper-Hewitt 110 volt, 4 ampere, mercury vapor lamp distant 20 cm. an exposure of 1 minute appeared to weaken it a little, while after 15 and 30 minutes all virulence was lost.

In two experiments the agent was killed by 0.05 per cent formol overnight at room temperature (22°C.).

Under the same conditions, 0.05 per cent phenol had no demonstrable effect, but 0.5 per cent abolished all activity.

Desiccation *in vacuo* after freezing weakened the agent but little, tissues so treated producing typical abscesses immediately afterward and at the end of a 45 day sojourn in the ice box. 5 rapid freezings at about −70°C., interspersed with thawings at 37°C., had no deleterious effect.

No loss of infectivity was observed in a saline extract stored in the ice box for 10 or 21 days, but at the end of 6 weeks the agent had become so attenuated as to cause rather small abscesses in only 2 out of 6 rats inoculated, and when tested after 78 and 8? days it was found entirely inert. Full virulence was retained by a ra? abscess kept in 50 per cent glycerol-saline for 31 days under the sam?

conditions of temperature, but there was some loss after 47 days and total abolition after 73 days. Infected mouse brain was virulent after cold storage in glycerol-saline for 32 days, but not after 45 and 60 days.

The agent is not susceptible to oxidation, under the conditions employed, the passage of filtered washed air through an extract at moderate speed for an hour having left its virulence unimpaired.

DISCUSSION

Relation of Filterable Agent to Tumor

During some 20 years' routine transplantation of rat sarcoma 39 no evidence of pus has ever been observed, purulent lesions having appeared only when the neoplasm had been treated in some way before inoculation, and then only from time to time. Thus abscesses followed the injection of tumor emulsions that had been incubated in tissue extracts prepared with Locke-Ringer or physiological saline solution, or with distilled water; of an emulsion that had been incubated in Locke-Ringer solution alone; and of one that had merely been combined with charcoal employed to adsorb antibody from tissue extracts. Apparently incubation, the various salts in the solutions, and the presence of a tissue extract were none of them a prerequisite, and the mystery was deepened by the fact that suppuration might appear in one of two experiments done under what were thought to be identical conditions and not in the other. A few preliminary efforts to elicit pus with sarcoma 39 under controlled conditions have failed. Emulsions of this growth incubated in various media, or oxidized by the passage of air, have produced no abscesses so far, but as the circumstances under which the pyogenic agent appears and reappears constitute one of its most interesting features a continuation of these endeavors may be worth while.

In any case there is no evidence so far to suggest a specific affinity between this agent and sarcoma 39, as has already been explained in discussing the text-figure. It is true that from 4 to 15 days after the agent had been injected into the blood stream or the testis in tumor-bearing rats it could be recovered uniformly from this sarcoma and from 4 other propagable new growths—sarcoma 8, the Walker carcinosarcoma 256, the Flexner-Jobling carcinoma, and a benzpyrene

sarcoma—but following either type of administration it was some-times demonstrable also in the blood, the liver, or the kidney. As neither these organs nor sarcoma 39 were found to contain the agent after subcutaneous inoculation, its transfer to them from the blood stream was probably an outcome of mere propinquity rather than of any definite attraction. Its uniform presence in tumors after intravenous injection, as contrasted with its occasional presence in the organs, is more easily explained by the favorable circumstances they provide for viruses in general than by any specific affinity between this agent and neoplastic tissue. Thus, for example, Levaditi, Schoen, and Reinié (5) have shown that rabies street virus will live in the cells of the Brown-Pearce rabbit carcinoma, Syverton and Berry (6) that the Shope papilloma can be infected with several viruses, and Rivers and Pearce (7) that the Brown-Pearce carcinoma will carry virus III and vaccinia virus, both of which survive there for a longer period than in normal tissues.

It may be mentioned in passing that the pyogenic agent could not be recovered from a Shope papilloma after injection into an ear vein of a rabbit bearing this growth.

When the agent was discovered in a tumor or an organ subsequent to intravenous or intratesticular inoculation it was not by virtue of any blood retained therein, for instances occurred where the agent was found in the liver, kidney, or neoplasm from an exsanguinated animal but not in the heart's blood itself.

The possibility of a cross-immunity between this filterable agent and sarcoma 39 has been investigated with some care. Rats that had recovered from an infection with the agent were discovered to be fully susceptible to the sarcoma, while rats that bore the tumor or had cured themselves of it and been proved refractory on second grafting showed no resistance to the filterable agent. Furthermore, introduction of the agent into rats with growing sarcomas had no visible effect upon their tumors.

Since the filterable agent shows no specific affinity for the neoplasm, and no immunological cross-relationship has been demonstrated be-tween the two, the only conclusion which can be drawn at the present time is that the agent is but a chance contaminant of the sarcoma.

One is then confronted by the question: How did the filterable agent

first gain access to the tumor? To the obvious reply: From the host, there are several objections. If sarcoma 39 is being contaminated from time to time through propagation in a widely infected breed of rats, many of these animals should be immune to the agent, yet the rat has still to be found that is refractory to the usual dose. Secondly, it would be a curious coincidence indeed if the occasional infected growth had happened each time to be employed for incubation or other treatment during the investigations on tumor immunity, and never for routine transplantation. In the third place, we have seen no evidence that this filterable agent can be passed spontaneously from rat to rat, at least in an active state. And, finally, it shows no disposition to invade the neoplasm even after a relatively enormous amount has been deposited under the skin and it has reached the lymph nodes, suprarenal glands, spleen, and brain.

If, on the other hand, the sarcoma became infected once and for all at some time in the past, and the filterable agent is now established permanently therein, why has it never declared itself during the 20 years throughout which this tumor has been cultivated? And, since it induces a fairly high immunity, why are rats bearing the sarcoma susceptible to the agent, when Rivers and Pearce (7) found that Brown-Pearce carcinomas infected with virus III or vaccine virus immunized rabbits against these two viruses? The lack of resistance cannot be explained by the assumption that the filterable agent remains imprisoned within the tumor cells, and therefore non-antigenic, since all cells except those at the periphery of the growth undergo necrosis as it enlarges, and because even rats in which sarcoma 39 has been completely absorbed are as susceptible to this agent as any stock rat.

The supposition that upon its original encounter with this neoplasm the filterable agent was lying latent in a rat, plausible enough at first sight, loses all force with the realization that even when the filterable agent has been disseminated throughout the host's organism it has no propensity to invade the tumor.

There is no difficulty in dismissing at once any proposal that the filterable agent may have entered from some outside source, for in 25 years' experience during which hundreds of routine transplantations have been performed with this neoplasm, the characteristic infection has never appeared.

Thus no explanation can be advanced which will fit all the evidence, unless one is prepared to consider the assumption of a filterable agent with duplex capabilities, able to induce inflammation under some circumstances and neoplasia under others. The situation in such a case would resemble that described by Andrewes and Shope (o -10), in which there was encountered an aberrant inflammatory rain of the rabbit "fibroma" virus. Another variant of this virus as been recorded by Berry (11), and many other viruses are known o undergo qualitative changes in pathogenicity without alteration of immunogenic power, the variant strains continuing to cross-immunize. As there was no evidence of cross-immunity between agent and tumor in the present instance, a mutation seems improbable.

It is conceivable that the pyogenic filterable agent herein described is one already known but now masquerading under an unfamiliar form in the rat. The virus of infectious ectromelia (12) produces swollen feet, but in this disease the paws are edematous at first and later gangrenous, never frankly suppurative. Furthermore, the virus of ectromelia is passed by contact, elicits extensive necrosis in the liver and spleen, is associated with the presence of abundant inclusions and the production of neutralizing antibodies, and is not pathogenic for the rat, all of which sets it definitely apart from the present agent. A thorough investigation of its immunological relationships and other features will be required before the latter can be assigned a final position among the infectious diseases.

SUMMARY

A filterable agent resembling the viruses is described. It was er countered in sarcoma 39, a propagable neoplasm of the white ra and has now been maintained in this species for 28 passages over period of some 7 months without appreciable loss in virulence. chief effect is the production of large abscesses in an animal spec comparatively resistant both to viral diseases and suppuration. white mouse is more susceptible than the white rat, the rabbit les and the guinea pig highly resistant.

The agent has been repeatedly recovered from sarcoma 39 tr in special ways, but under the ordinary circumstances of ro

transplantation it does not manifest itself. As yet there is no certainty on where it came from or how it maintains itself under natural conditions.

We are indebted to Miss Hede J. Frank, who did much of the preliminary bacteriological work, to Miss Eleanora Molloy, who cultivated the agent in the egg, and to Dr. John G. Kidd, Dr. Thomas M. Rivers, and Dr. Albert B. Sabin, all of whom have given generously of their time to review our evidence and examine our material.

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EXPLANATION OF PLATE 17

FIG. 1. A 4 day abscess, showing the thick wall and the congestion in the adjoining subcutaneous tissue.

FIG. 2. An 11 day abscess, consisting of a thin walled pus sac. Below and to the left a smaller abscess is seen.

FIG. 3. Suppurative lesions of the forepaws in a rat inoculated 14 days previously in the testis.

FIG. 4. Necrosis and polymorphonuclear infiltration 24 hours after intradermal inoculation. At the lower edge of the photograph appears the margin of a large abscess. Hematoxylin and eosin. ×150.

FIG. 5. Cytoplasmic inclusion in epidermis overlying a 1 day intradermal abscess. Eosin and methylene blue. ×600.

FIG. 6. Cytoplasmic inclusion in epidermis overlying a 1 day intradermal abscess. Eosin and methylene blue. ×1600.
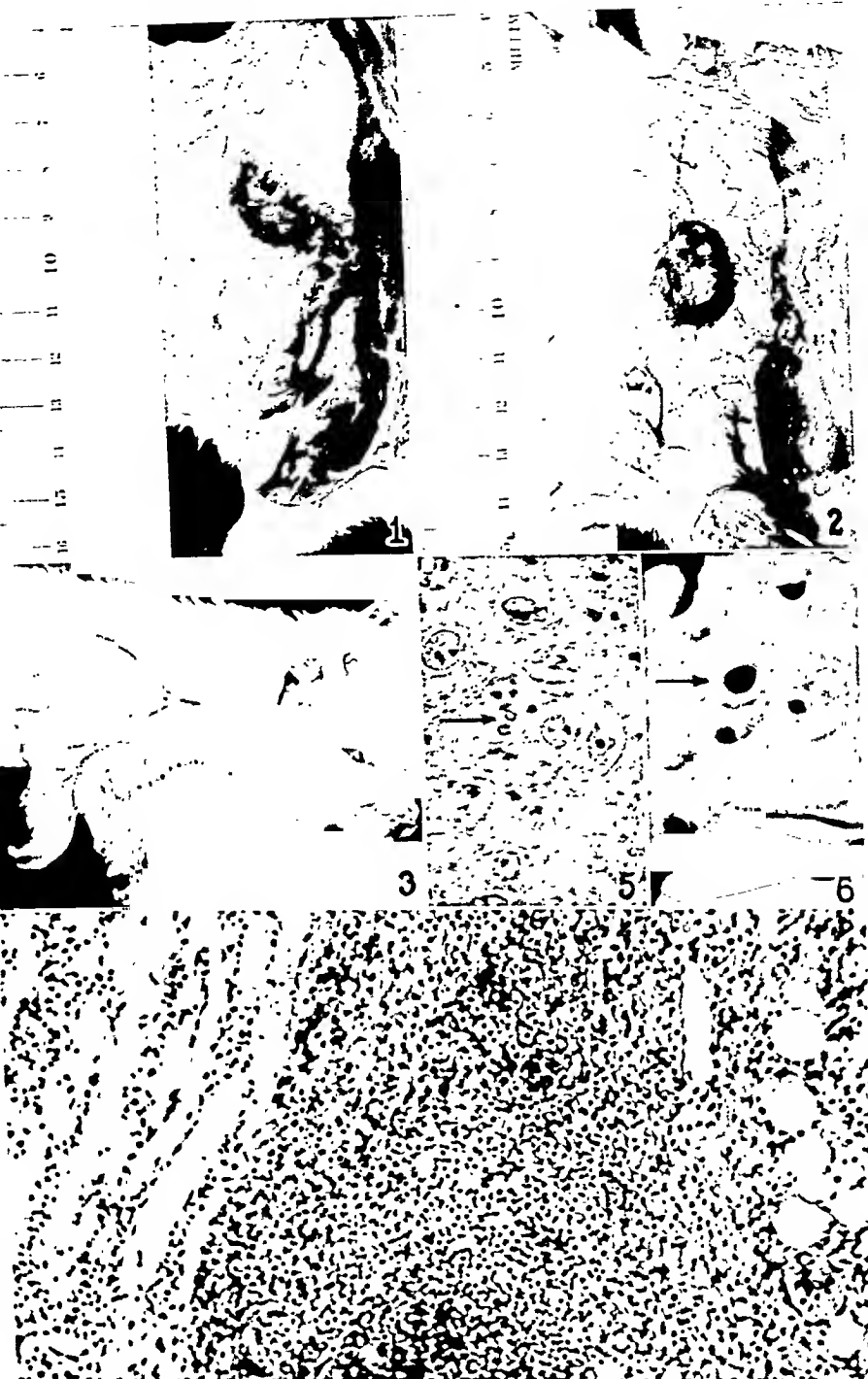
THE CARCINOGENIC EFFECT OF A PAPILLOMA VIRUS ON THE TARRED SKIN OF RABBITS

II. Major Factors Determining the Phenomenon: the Manifold Effects of Tarring

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PLATES 18 TO 23

When the virus causing rabbit papillomas (1) is injected into the blood stream of rabbits repeatedly tarred on the ears it often localizes abundantly in these organs and gives rise there to carcinomas (2) and papillomas differing from the ordinary. The general course of events has been described in a preceding report (3). The present paper deals with the factors responsible for the unusual tumors, and with the influence of tarring to render the virus effective, to further proliferation of the growths and, in not a few instances, to make them anaplastic. The experiments and charts will be numbered consecutively to those already published.

The Experiments

So much virus localized in the tarred ears of our first experiments (Experiments 1 to 3) that confluent proliferation usually resulted, which soon filled the aural concavities with foul, fungoid tissue, death occurring early from sepsis. The individual growths could not readily be observed in their early stages, because the tarring was continued while they were appearing, and when it was left off later they often had coalesced or were crowded together. To obviate these difficulties, a smaller inoculum was employed in some of the present tests, to produce scattered, discrete growths, and most of the rabbits were not tarred after inoculation.

General Method of Recording the Changes.—The growths were drawn to size in their relative position, on stamped outlines. Preliminary records were made of

529

the tar warts, when any existed, and after the virus injection the tumors were charted every few days, the interval depending upon the rapidity of the changes. Notes were taken on all growths especially worth attention.

The size of the ears often varied from the average employed for the stamped forms, and then the tumors as drawn were crowded or scattered more than in life. Dubious growths are indicated by a broken line. The sudden appearance of rapidly enlarging, dark gray or black papillomas provided a tell-tale to infection with the virus, since pigmentation is infrequent in tar tumors and is retained only while they are indolent (3). Hence melanotic growths are charted in black. Confluent masses are merely outlined unless melanotic. Indubitable cancers are recorded in red, from the day they became visible as tumors. The stippling around the border of certain growths of Chart 11 that were included within large melanotic masses indicates uncertainty as to how far they extended.

The protocol of Experiment 4 has already been summarized (3), and the cancers described.

Experiment 4.—Brown-gray ("agouti") rabbits were used, as in every test, and tar of the Oster-Gasfabrik of Amsterdam, kindly provided by Dr. Landsteiner. The 38 animals had been tarred twice weekly for 89 days on a central area of the inner surface of the ears, about half their total expanse, whence spreading occurred to the edges. Before every third application the layer was stripped away. At the stripping 24 hours before inoculation, the "tar warts"[1] were charted, the state of the ears recorded, and the animals were separated into 4 comparable groups. 3 of these were injected into a leg vein with 15 cc. each of a Berkefeld filtrate of a Tyrode extract of glycerolated papillomas (W. R. 1183), 2 groups receiving a filtrate of 0.5 per cent strength (in terms of weight of tissue extracted), and the third the same fluid diluted with Tyrode to 0.2 per cent. An hour later this last group, 11 animals, were tarred again, as were also the 10 controls and the 8 rabbits of one of the groups receiving 0.5 per cent filtrate; and the tarring was kept up twice weekly throughout the next 25 days. The 9 animals of the second group injected with 0.5 per cent filtrate were not tarred again.

Within 15 seconds after each injection, a circular area 2 to 3 cm. across on the shaved right side was tattooed with the needles of an electrical tattooing machine, sterilized by heat, and a similar tattooing was done on the left side, but through a few drops of the inoculum. The needles brought blood. Our aims were respectively to determine whether the virus would localize from the blood in traumatized epidermis, and to produce papillomas as an index to susceptibility. At no spot injured with sterile needles did growths develop; but where the inoculum had been tattooed in they promptly appeared save in one

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[1] In Paper I the morphology of the tar warts, actually tumors, has been summarized. Their histology will be dealt with subsequently.

instance in which an abscess formed instead. Susceptibility in this case was attested by the appearance of a growth where the skin had been punctured for the intravenous injection. All of the papillomas remained merely such.

The ear changes are recorded in Charts 6 to 9. The records dealing with the outsides, which had epidermis but slightly changed, are not reproduced since only ordinary virus papillomas arose there, nearly all dark gray. The ears of 3 controls and 1 animal receiving 0.2 per cent virus are omitted because they remained devoid of growths. Those of one animal receiving 0.5 per cent virus and tarred later, and of 2 not tarred again, are likewise omitted, there being no evidence of virus localization in their ears, which were little changed by the tarring and carried no warts or only one or two.

In several of the *controls* (Chart 6) the later tarring elicited a few new warts, but after it was discontinued some dwindled and disappeared, others remaining stationary, or enlarging slowly. None ever appeared on the outsides, no rapidly enlarging ones on the insides, and no cancers. Most of the animals were kept many months after the experiment ended, the majority of their tar tumors vanishing, and the others becoming indolent.

So few tumors followed *injection of 0.2 per cent virus with later tarring* (Chart 7) that the group serve as additional controls. Where the inoculum was tattooed into the body, papillomas appeared late, as always with dilute virus (4). In 5 individuals 1 or more sooty, rapidly enlarging papillomas developed on each side of the ears after a few weeks, while in two others they appeared on the insides only; but a mere scattering was obtained at best. In one individual, however (No. 9, Chart 7), most of the preexisting warts disappeared when tarring was left off, but a few began to grow rapidly when the virus took effect, as evidenced by dark gray papillomas on the outside of the ears, and they soon outstripped the growths of any control. Nothing further of significance took place and the group was discarded on the 57th day.

In several rabbits *injected with 0.5 per cent virus but not tarred later* (Chart 8), a considerable number of growths arose in the 3rd to the 6th week after injection. Those on the outside of the ears were ordinary, conical or onion-shaped, virus-induced papillomas, nearly all dark gray, whereas many on the inside were pink. Here, however, other pink growths appeared concurrently that were discoid and fungating, and in several instances these enlarged with a rapidity unexampled in the controls, and so too did some of the preexisting tar warts. In rabbit 20 which died on the 57th day, one of the latter which had taken this course reached enormous size and like 3 others in the same animal, all originally tar warts, had the histology of an anaplastic carcinoma. Yet since none metastasized or caused extensive destruction, they cannot be set down as cancerous.[2]

In 5 of the 8 rabbits *receiving 0.5 per cent virus, and tarred later* (Chart 9),

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[2] The distinction to be made between carcinomas and carcinoids,—growths which look like cancers though incapable of independent progression, is discussed in Paper I.

532

Days after injection

Rab No.	At injection		22		30		36		43		57		71		85	
	R	L													
18															
19															
20															
21															
22															
23															
24															

A Abscess: but pap. grew on leg.

534
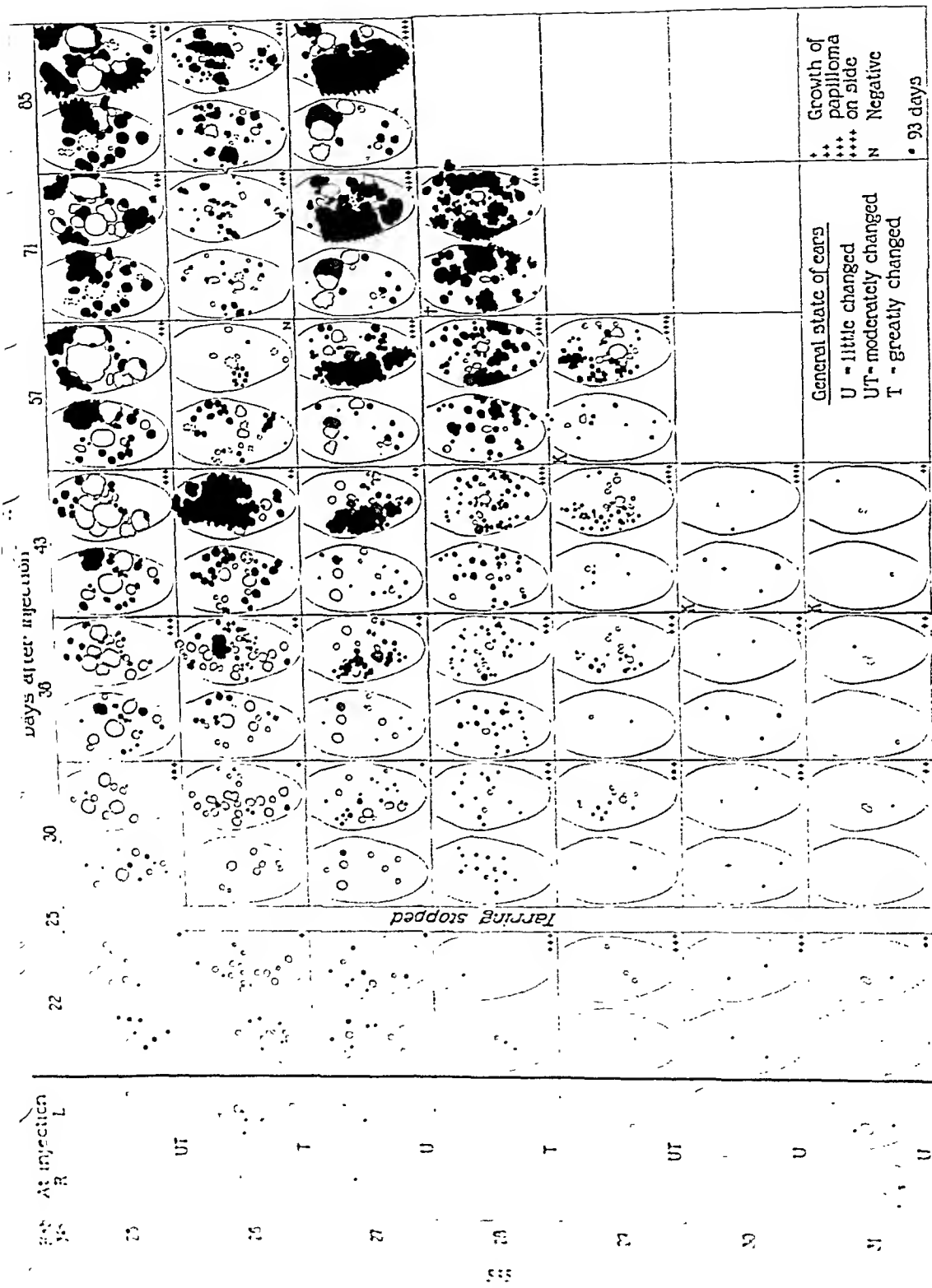
CHART 9.   0.5 per cent virus: tarring kept up.

many rapidly enlarging papillomas arose, and the influence of the virus was attested both by the great contrast with the controls and the frequency of melanosis.   Vigorous pink growths of the various sorts described in Paper I appeared concurrently, and in 2 of the 5 rabbits in which the virus proved most effective, as evidenced by tumors (Nos. 27 and 28), some proved carcinomatous.   Rabbit 28, with a single cancer, died early of intercurrent causes, but rabbit 29, with 4 malignant growths, lived long enough for huge metastases to form on both sides of the neck (3).

In this experiment the tar tumors of the control animals behaved as usual after tarring was left off, many disappearing and the rest remaining stationary or enlarging slowly as benign growths (Chart 6). One group of animals receiving virus got so little as to cause only a few new tumors, and these proved ordinary papillomas (Chart 7). This group were tarred for 25 days after injection.   In the group receiving four times as much virus but not tarred thereafter, more growths appeared (Chart 8), and their variety was greater, but while some looked cancerous there was no certainty in the matter.   In a group similarly inoculated and tarred for 25 days, the virus proved notably effective, causing a multitude of growths in many instances and carcinomas in some (Chart 9).

In the next experiment more virus was injected and the attempt was made to prevent it from localizing in one of the ears.   To this end the circulation to the organ was obstructed for some minutes after the intravenous injection was begun.

Experiment 5.—Tarring was done twice weekly for 84 days before the injection. The ears of the 28 rabbits were charted on the 81st day, with no more tarring until after the injection.   The animals were separated into 3 comparable groups, 2 of which received 15 cc. of virus fluid intravenously, the third serving for control. The inoculum was a Berkefeld filtrate of a 5 per cent Tyrode extract of the pooled, glycerolated papillomas of 8 cottontails.   Just prior to its injection into a leg vein,—which required about a minute,—a rubber band was wound tightly around the base of the left ear and not cut away until 2 to 22 minutes later.   To determine individual susceptibility virus fluid was tattooed into the left side of the body, as in Experiment 4.   The ears of the controls and one injected group were tarred again after 22 hours and twice weekly during the succeeding 21 days.   To the other injected group nothing further was done.

A few additional warts appeared during the later tarring of the 9 *controls* (Chart 10), but afterwards the majority vanished, though some remained sta-
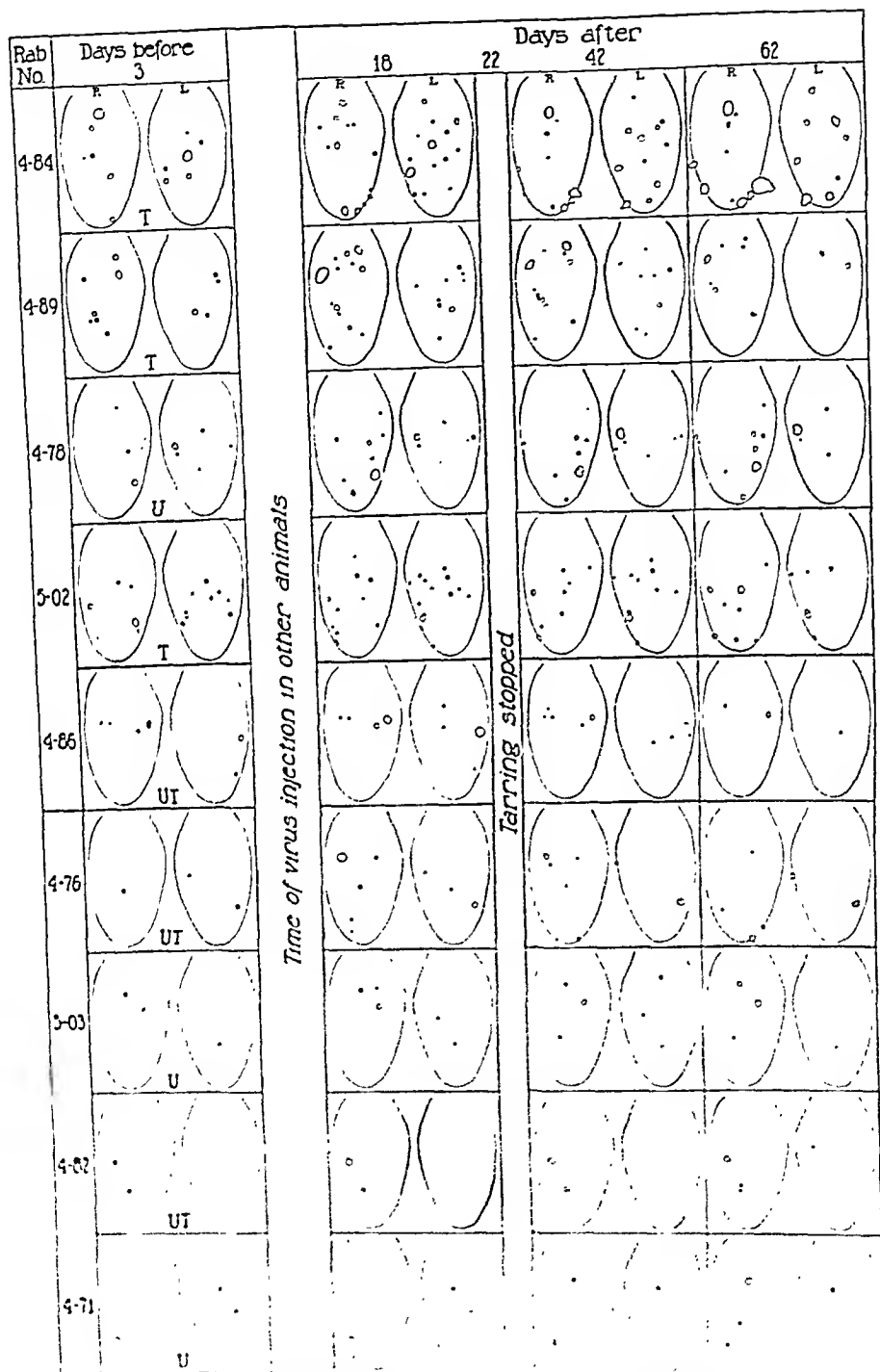
CHART 10. Tarred controls: no virus injected.

CHART 11.— Virus injected: no tarring thereafter. Growths on insides of ears. Some cancers have not been charted because overlain by melanotic papillomatous tissue. [This was frequently true of those which had extended to the outside of the ear.]

tionary or grew slowly throughout the 62 days of observation. The rabbits with most persisting warts were kept many months, and nearly all of their growths retrogressed, a few remaining stationary or very slowly enlarging. No cancers ever developed.

All of the injected rabbits proved susceptible, papillomas developing at the tattoo site. The 8 animals *injected and tarred later* yielded such heterogeneous findings that no comparison can be made with those not tarred again. In 1 no growths indicative of virus localization arose, and in 2 only a few sooty papillomas. In 2 others more growths of this sort were elicited but most of them disappeared after some weeks, as did all the tattoo papillomas. In 1 of the remaining 3 a moderate number of progressively enlarging growths appeared, but none had a malignant aspect when the rabbit was discarded after 106 days. In the other 2 a great variety of tumors developed, cancers amongst them. They illustrated special points, to be handled in a succeeding paper.

The ears of 2 of the 11 rabbits *injected but not tarred again* were but slightly changed, and the virus caused merely a few dark gray papillomas. Several pre-existing tar warts started to grow rapidly, but none seemed malignant and the rabbits were killed early, with no blocks taken. In a third animal a sparse scattering of growths arose on the ear submitted to the tourniquet for 14 minutes, while they were fairly numerous on the unobstructed one; but again they promised little and the rabbit was discarded. Charts 11 and 12 record the changes in 6 other animals, killed between the 45th and 62nd days. The virus-induced growths were relatively few in one instance, no cancers were recognizable amongst them, and the animal was discarded early. The ears of another were saved in the gross: they carried several tumors with the aspect of cancers, but in the lack of histological confirmation none was recorded as such. Multiple carcinomas appear in all of the other 4 and a glandular metastasis in 1.

The 2 rabbits which do not appear in the charts were kept several months longer. The virus elicited numerous cancers on their ears, with a glandular metastasis in one case. Bits of the metastatic tumor were transplanted to the leg muscles with result in growths.

Cutting off the circulation for 2 minutes (Charts 11 and 12) was not followed by a noteworthy difference in number of growths elicited, but $4\frac{1}{2}$ minutes decreased it, and 20 to 21 minutes reduced it greatly. Under such circumstances the ear submitted to obstruction served usefully as a control. The number of carcinomas was considerably greater than the charts indicate, since only those are recorded which were examined microscopically, and this was undertaken with less than half of them.

The individual findings will be summarized. The pathological

changes due to tarring rabbit ears have been the theme of many investigators (7), and those noted under the circumstances of our experiments have already been described (3). There was much individual variation in hyperkeratosis, connective tissue proliferation, vascular disturbance, etc., as well as in the incidence of tar warts.

No. 5-12: Ears almost unchanged by the tarring. All of the virus-induced growths were sooty papillomas. Discarded after 45 days.

4-75: Ears moderately changed: several tar warts. After the injection a fair number of sooty and pink growths appeared on the ear submitted to the tourniquet, a scattering on the other ear. Killed on 51st day. Of 8 pink growths sectioned 6 proved cancerous, the others benign papillomas.

4-95: Ears much changed: few tar warts. Many new, rapidly enlarging pink growths appeared in the 3rd to 5th week after the injection, and toward the end of this period great multitudes of minute, sooty ones, which soon coalesced. Both were especially numerous on the ear submitted to the tourniquet. Killed on 59th day. Of 7 pink growths sectioned 4 were cancerous. One of these was primary on the outside of the ear. Another extended through to this side and became large here.

5-13: Ears much changed: many tar warts. The happenings were like those in 4-95, the ears differing even more. Killed on 54th day for gross specimens; no blocks.

5-07: Ears much changed: several large tar warts. One on the right ear began to grow fast soon after the injection and became a broad cancer. The general course of events was as in 4-95, 5-13. On the 54th day the left auricular lymph node was discovered to be much enlarged and nodular, and at sacrifice on the 61st day it was 1.8 cm. across, almost wholly replaced by a cystic, malignant papilloma. At no time had the ear been incised. One of the large tar warts became malignant after the injection. Of 7 pink growths sectioned, 4 were frank carcinomas, the others papillomas of diverse character.

4-72: Ears little changed: one small tar wart which remained stationary later. Same happenings as in rabbits just dealt with: killed on 61st day. Of 10 pink growths sectioned 6 were cancerous, 1 primary on the outer side of the ear. Several other carcinomas had been removed by early biopsies.

The 2 individuals not included in the chart were let live until the 137th day. They too developed multiple cancers, which at death occupied most of the ears. These had not been cut into at any time. A metastasis was discovered in an auricular lymph node of one of them (D. R. 4-73) on the 76th day, and portions were implanted in the muscles of all four legs of the host on the 90th day, with result in masses of invasive squamous celled carcinomatosis, 1.5 to 3.0 cm. across at autopsy.

Increasing the amount of virus had the desired effect in this experiment; it localized abundantly in the ears and rapidly elicited a variety

of growths, many carcinomas amongst them.   Later it also produced hosts of ordinary gray and pink papillomas which remained benign: a large number were sectioned incidentally to study of the unusual tumors.   The virus was obviously responsible for the latter, since none appeared in the controls, despite the later tarrings, and relatively few on the ears submitted to the tourniquet.

The experiment demonstrated that tarring after injection of the virus is not essential to the production of carcinomas.   In the present instance as many appeared in the animals no longer tarred as in the most favorable of the tarred group.

The main object of the next test was to learn whether virus materials from different sources would give rise in the tarred skin to differing growths.

Experiment 6.—The 20 rabbits had been tarred twice weekly over a period of 27 days, once more on the 37th day, and again on the 40th day, 4 hours prior to the intravenous injection.   The changes thus induced in the ears were less pronounced than in any previous test, yet in some instances these organs carried a few tar warts.   The animals were separated into 4 comparable groups and injected intravenously with one or another of four highly pathogenic virus fluids deriving respectively from the glycerolated "spontaneous" papillomas of 3 wild rabbits and from the pooled material utilized in Experiment 5.   5 per cent Berkefeld filtrates were injected, 15 cc. for each animal, and some of the inoculum was tattooed into the side of the body.   Here it gave rise to papillomas only.

All of the rabbits proved susceptible to infection, and, on the ears of many, multitudes of growths arose after the incubation period usual with the virus. The general course of events was precisely like that in Experiment 5, with cancers nearly as numerous.   The ears had been tarred less than in any preceding experiment and were not tarred after the injection.   The character of the tumors yielded no indication of qualitative differences in the inocula.

A further experiment had as its aim the microscopic study of early stages.   The ears were briefly tarred, infiltrated directly with large quantities of virus, and soon after the growths had shown themselves some were punched out.

Experiment 7.—The 3 rabbits had been tarred twice weekly for 28 days, and had ears slightly to moderately changed, with no warts, one small wart, and 2 warts respectively.   After the usual stripping and charting the left ear was warmed to distend its vessels, a rubber band was wound tightly around its base,

and by way of a marginal vein it was infiltrated with 10 cc. of 1 per cent virus fluid, derived from the pooled papillomas of 3 experimentally inoculated wild rabbits, and centrifuged water-clear. As the fluid went in the ear became turgid and slight oozing took place from cracks in the hyperkeratotic epidermis, and from the tar warts especially. Leakage from the wound made by the injection needle was prevented with a metal clip. 30 minutes later the rubber band was cut away, after another had been placed about the base of the other ear. This was left on 30 minutes to prevent localization of the virus flushed out of the infiltrated organ when the blood entered it again.

A profuse virus infection took place, especially in the animal with no tar warts. Its infiltrated ear suddenly became thick, hot, and brawny during the 2nd week, a change noted in previous experiments (3) when much virus had localized in the tarred epidermis, and a multitude of growths soon arose, on the injected, outer side especially. After 14 days they were fairly numerous, discrete, ½ to 3 mm. in diameter; and soon they appeared in a crowded horde, coalescing and covering the entire surface. The control ear remained thin, cool, and devoid of tumors. In the other rabbits no brawny thickening occurred, and not so many growths developed. In each case the ear injected carried the tar warts. The 2 present in one animal began to enlarge rapidly when the virus-induced tumors were appearing, and after several weeks proved cancerous. The innumerable growths elicited by the virus manifested some of the variety noted in previous experiments, but only a few were malignant. Most of these were punched out within a few days after their appearance. The control ears reverted toward the normal in one case, remaining free from tumors, while in the other two small growths arose, a pink one with the distinctive morphology (later to be described) of a tar papilloma, a second sooty, and obviously due to the virus. The rabbits were killed after 27 to 34 days.

The Anomalous Tumors

A comprehensive description will now be undertaken of the *anomalous tumors*, as one may term all those growths elicited by the virus in tarred skin, which differ from the standard productions (benign papillomas) characteristic of its ordinary action.

The papillomas which follow upon direct inoculation of the virus into the scarified normal skin of "agouti" rabbits are remarkably uniform in morphology, though some are pink and the majority gray or almost black. The difference is due to melanoblasts, passively included in the growths, which multiply and form abundant pigment, though they do not become neoplastic (5). They are most likely to be included when the papilloma is the outcome of proliferation at many neighboring points; and, since multicentric growths enlarge most rapidly, it follows that those which first become visible are most likely to be melanotic.

Nearly all produced by an inoculum of high titre are composites of cell families, each the outcome of a cell-virus association: and certain families may proliferate with special vigor and aggressiveness, pushing others to one side, extending beyond the included melanoblasts, and forming discrete, non-pigmented nodules in the midst of the mass and sometimes beneath it (6). Usually, nevertheless, all of the papillomas of any one rabbit run the same general course, enlarging together, and perhaps all retrogressing secondarily. The fewer the cell families of which they are constituted, the more likely are they to differ in small ways.

When *normal* rabbit ears are directly infiltrated with virus fluid as in Experiment 7, and then tarred several times, numerous growths may appear, all ordinary virus-induced papillomas and the generality punctate in origin. The differences they manifest when tarred do not exceed those just described, the uniformity in size, shape, and behavior being remarkable. The growths on the outside of the ears, where melanoblasts abound, are mostly dark gray, whereas those near the middle of the inside may be pink, with gray ones frequent toward the edges. Histologically the two differ only in content of melanoblasts. If a piece is punched from a spot covered with gray growths, while these are still appearing, an enlightening phenomenon may follow. Papillomas wholly devoid of melanosis arise from the epithelium that has extended to cover the raw edges of the punch hole, and enlarging with special vigor, they soon protrude from amidst the host of gray growths as pink cauliflower rosettes or hassocks (Figs. 1 and 2). Yet they have the usual histology,—though staining lighter with methylene blue,—and it is plain that they owe their lack of pigmentation to origin from an epidermis which has extended farther than the melanoblasts, and their rapid enlargement to the favorable conditions provided by the healed wound. Usually they soon become stalked, doubtless because of the ductility of the new connective tissue (Fig. 2).

Two or three tarrings of the ears prior to an intravenous injection of virus will cause it to localize in them, but only ordinary papillomatosis ensues. Many of the growths must be the outcome of individual cell-virus associations, yet their general sameness is remarkable, especially when thousands are present. If the preliminary tarrings have been many, the skin on the neck where the ears rub may have become hairless and hyperkeratotic; and here the virus may localize and cause papillomas of ordinary sort, which grow or retrogress together, with negligible exceptions.

The first evidence that preliminary tarring results in different kinds of virus tumors was obtained when the ears had been tarred during 4 weeks and were then directly infiltrated with virus fluid (Experiment 7). Pink growths arose some days before any gray ones did so,—the reverse of the usual occurrence,— and they grew much faster than the latter which usually appeared in relatively immense number. Tarring for periods up to 3 months, with intravenous inoculation of the virus and no more tar (Experiments 5 and 6), resulted in the same

occurrences in more pronounced form, the incidence of the unusual pink growths being greater. Most became visible during the 2nd to the 4th week after virus injection, almost none arising afterwards, whereas the ordinary gray papillomas kept on appearing until the 8th to 12th week, and often became a crowded host. All of the growths were subepidermal mounds when first noted, but whereas the anomalous pink tumors frequently reached a diameter of 3 to 5 mm. prior to erupting, the gray papillomas were seldom more than a millimeter across before they did so. The difference was not one of origin, for the majority of both sorts derived from the epithelium of hair follicles rendered cystic by retained keratin: it lay in the activities of the virus-infected cells. Those constituting the ordinary papillomas, though stimulated to rapid multiplication, heaped up in the direction of least resistance, which was outwards, whereas the cells of the anomalous pink growths were more aggressive, and extended laterally for some distance before the mass erupted. Yet some of the pink growths proved to be mere Shope papillomas, or variants thereof (Fig. 25), though as a rule staining lighter with methylene blue than their gray fellows. Other pink growths exhibited distinctive morphological differences from the first (Figs. 4 to 9), the folded, subsurface layers of proliferating epidermal cells being compact, cystic, or convoluted, and taking the methylene blue poorly. Nevertheless the influence of the virus was still manifest in the cytology of many. The pink growths were situated mostly near the middle of the insides of the ears, where the tar had produced greatest change, but occasionally on the hyperkeratotic, outer side also (Chart 12, rabbit 4-72); and beginning as mounds, they rapidly became hemispheres while still subepidermal, and, sometimes within a few days, took the form of deeply embedded, fleshy globes covered with tense, glistening epidermis (Fig. 3). Often, they scabbed at the summit before this happened. Some grew rapidly, some slowly, but nearly all soon dried at the top and enlarged into plump cones or "onions" or jagged masses (Figs. 10, 19), whereas the ordinary gray and pink papillomas arising later and growing nearby were, by contrast, keratinized nearly to their bases in most instances and became dry, jagged spires, horns or cauliflowers, confluent next the ear surface, or discrete even where greatly crowded. As the pink growths rose higher their dry peaks turned dirty gray, and when their bases were hidden the hue was easily mistaken for genuine melanosis. It follows that the latter was not so prevalent as some of the charts would imply.

The anomalous growths of malignant sort which arose on ears no longer tarred, appearing as pink mounds like those already described, usually enlarged into embedded spheres, scabbing or drying at the top. A few ulcerated early, and became discs with raised, infiltrating edges (see Paper I), or formed scabbed, fleshy hassocks; but the majority built up into fleshy, broad-based cones or onions, often vertically ribbed, and sometimes with such a superficial resemblance to benign papillomas that their true character was overlooked until metastasis had occurred. When cut into, though, even at an early stage, their irregular markings, punctate necroses, and extension downwards and sideways disclosed

their true nature. Frequently they broke down into ulcers and then built up again. When they extended through the ear, a frequent happening, they formed firm mounds on its outer side, which either became ulcers with thickened, infiltrated edges, or else low, fleshy cones which broke down and built again, perhaps repeatedly. Some of cystic character formed subepidermal aggregates of partially coalesced nodules from which firm prongs with keratinized cores extended toward the head, often within the large lymphatics. (*Vide* the cancer of rabbit 28, Experiment 4 (3).) Frequently the rabbits kept the malignant tumors abraded and bloody, as if they caused annoyance. As they got bigger they became broad, weeping, fungating masses, or ulcers almost at the skin level (Paper I). Some metastasized early to the regional glands (Figs. 11 and 12), but others failed to do so though occupying almost the whole ear. The metastases were frequently cystic,—in which respect they resembled some tar cancers (7), as also some carcinomas developing secondarily from ordinary virus-induced papillomas (8). The implantation of bits of the metastases in the leg muscles of the host resulted in carcinomatous masses with a tendency to greater anaplasia (Fig. 13). Many of the rabbits of Experiments 5 and 6 carried 5 to 10 or more large cancers on each ear when they were killed, 2 to 4 months after inoculation. Occasionally one arose on the hyperkeratotic outer side, as did also benign anomalous tumors. Not every threatening growth was sectioned. All of proved malignancy were non-melanotic, another point of similarity to tar carcinomas and those deriving secondarily from Shope papillomas.

When tarring was kept up after virus injection, ulceration soon took place of many of the benign growths as well as the malignant and they grew fast, and could not always be told apart for some time. Not infrequently the continued tarring also elicited tumors referable to it alone, and the longer the procedure was kept up the more often they occurred. Under such circumstances it sometimes proved impossible to say of a given tumor in the gross whether it was of tar or virus origin; yet when many new growths arose in the injected group and few or none in the controls, or on that ear of injected individuals which virus was prevented from reaching (Experiment 5), one could be certain that it was responsible for the generality.

Most of the pink, anomalous growths of ears no longer tarred retained their initial advantage over the crop of ordinary papillomas that arose later. The latter while small often resembled strewn iron filings set on edge with a magnet, the pink growths standing forth from amidst them as subepidermal globes. Later they rose into crowded, high peaks, confluent at the base; and now their competition with the pink growths affected the latter adversely, because of their great number and prodigious energy of proliferation, which often far exceeded that of the associated carcinomas, these triumphing by the destruction they caused. Frequently a jagged mass of gray, confluent papillomatosis pressed upon and overhung the malignant ulcers, obscuring them until a patch of the mass or the

entire end of the ear suddenly came away, exposing large cancers which had been burrowing and destroying all the while. Those which had become fleshy cones or hassocks were sometimes so squeezed by the surrounding papillomatous tissue that they dwindled amidst it while extending beneath. When they grew through lacunae in the cartilage to the outside of an ear covered with ordinary papillomatosis they sometimes remained invisible until the ear was sliced at autopsy. This held true of a tumor of Chart 11, which hence finds no record in the charts made during life.

The successive charts of the same ear often disclosed great individual variations in the course even of typical Shope papillomas. For example in rabbit 10 of Chart 7, 6 sooty papillomas disappeared from the left ear between the 43rd and 57th day, while a seventh grew and a wholly new one appeared and enlarged rapidly. In rabbit 9 of Chart 8 several dark gray papillomas disappeared from the middle of the right ear between the 57th and 71st days, as did also one at the injection site on the leg, whereas the other growths of this sort on the ears continued to enlarge. Later nearly all of these disappeared, a few continuing to enlarge during many further weeks, as shown in charts not reproduced. In rabbit 15 of Experiment 1 (3) most of the ordinary melanotic papillomas disappeared from the ears, yet some continued to grow, as did all of the cancers and certain pink papillomas.

The course of the anomalous growths varied notably. While nearly all appeared soon after the injection, an occasional one came late, after 2 months or more. Some of the benign ones retrogressed while others enlarged concurrently. After tarring was left off the malignant tumors generally progressed, though handicapped by the rapid reversion of the ear tissues toward the normal. Some grew fast, some slowly, and very occasionally one vanished after progressing and destroying for many weeks.

So sharp was the contrast between the pink anomalous tumors elicited in Experiment 5 and the ordinary virus-induced papillomas, gray and pink, which arose later (Figs. 3 to 10), as to suggest the presence of two differing sorts of virus in the composite inoculum. True, this had caused only ordinary papillomas where tattooed into the skin. Nevertheless some rabbits were inoculated with serial dilutions of each component of the material, these being still available. There resulted only papillomas. Comprehensive observations, made later, upon the effect of other virus materials (Experiment 6) justified the conclusion that the variety of the neoplasms was due to differing changes in the epidermal cells affected by the preliminary tarring, not to peculiar virus constituents.

Numerous early biopsies were made to learn how the anomalous tumors arose. Often these were made at a time when the ordinary papillomas had only begun to appear, and whenever possible growths of both sorts were included in the same microscopic section (Figs. 4 to 9).

The histological findings were easiest to interpret when no further tarring had been done. Under such circumstances almost all of the anomalous growths consisted in their earliest stages of irregularly folded layers of stratified epithelium from which tongues and strands of cells often extended down. From the first all were distinctively different from ordinary virus papillomas, the differences being relatively slight in the case of benign growths, finding expression ordinarily in thicker epithelial layers, more complex pattern, less tendency to keratinize, and lighter staining with methylene blue. Those malignant tumors which were least anaplastic retained the papillomatous form but had convoluted layers of epithelium, lying in a loose, perhaps spongy, disorder. The jumbled cells colored palely with methylene blue (Figs. 6, 8, 18), and often died before keratinizing. Such growths invaded the underlying tissue from the beginning (Fig. 8), as squamous cell carcinomas which sometimes formed keratinized cysts secondarily (Fig. 19). There were many minor differences in the histology of the individual growths. Very few of the untarred cancers were markedly anaplastic in their early stages, whereas this was usual with those tarred (see Paper I). The coalescence of neighboring growths often yielded complicated findings.

Many of the cancers retained their initial morphology, while others underwent further disorganization, and yet others soon became wholly anaplastic (Fig. 14). Tarring greatly hastened these changes.

The histological features were most readily interpreted when the virus had localized at scattered points, giving growths of unicentric origin. In Experiments 5 and 6 many were multicentric, owing to the large inoculum, and their composite character was frequently evident in the gross, some being spotted, streaked, or segmented in gray and pink. Microscopically in such cases ordinary virus papillomatosis was often joined to, or intermixed with, anomalous proliferation, both incorporated in one mass (Figs. 16 and 17). Sometimes the pattern of the keratinized material made clear the fact that the differing neoplastic components had long been present (Fig. 17). Such mixed growths usually became cancerous everywhere within 2 or 3 months, their malignant components destroying the benign.

The greater the preliminary changes due to tarring the more often, generally speaking, did the virus cause anomalous tumors. There were notable exceptions, however, the virus in certain instances causing many anomalous growths, some malignant, in skin but slightly

changed, and in others producing none despite marked cutaneous alterations. Most of the experiments were carried out on animals tarred from 40 to 90 days; and we have gained the impression that the longer the period, within these limits, the more frequently were the anomalous tumors malignant. Since the incidence of tumors due to tarring, as such, is greatest in skins that undergo most change, it follows that ears carrying many tar tumors were those in which anomalous tumors were most prone to develop after the virus injection. Yet once more exceptions were met, some ears devoid of tar tumors yielding many anomalous growths in response to the virus (rabbit 4-72 of Chart 11, left ear), whereas others with large ones developed only ordinary papillomas.[2]

Influence of the Susceptibility of the Host

In most of the experiments some of the inoculum was tattooed into the side of the rabbit. All thus tested proved susceptible, but in widely differing degree, the tattoo growths appearing late and sparsely in some, and in others coming early and in great numbers, soon assuming confluent form. It was in individuals of the latter sort that the cancers appeared. Yet pronounced susceptibility combined with a profuse localization of virus in tarred skin did not suffice of themselves to insure the occurrence of malignant growths.

In rabbit 6-51 of Experiment 3, injected after long tarring, the epidermis underwent a broadcast infection with the virus, and the ears reached prodigious size; yet careful search disclosed no cancers. The virus caused a multitude of papillomas in rabbits 25 and 26 of Experiment 4 (Chart 9), and by the 30th day they had outstripped those due to it in rabbits 27 and 28; yet cancers appeared only in the latter animals. Rabbit 13 of Experiment 1 bore a host of vigorous, virus-induced papillomas but no malignant tumors, whereas rabbit 15, with few papillomas, and these mostly slow to appear and enlarge, developed several carcinomas.

Not infrequently after the virus papillomas had prospered for weeks most or all retrogressed rapidly and disappeared (rabbit 26, Chart 9),

[2] The injection of a highly potent virus into the 2 animals of Experiment 3, with large tumors due to tarring for 40 weeks, resulted in but a single cancer amidst massive papillomatosis.

together with the tattoo papillomas and any that had arisen at the injection site (rabbit 19). Some generalized host influence was obviously at work (9). Many of the benign, pink, anomalous tumors likewise vanished, but others kept on enlarging, and so too did certain of the ordinary virus papillomas, in not a few instances (rabbit 15, Experiment 1). The cancers continued to progress but no new ones arose.

Influence of the Amount of Virus Infection

Very slight alterations due to tarring will bring the virus out of the blood stream, but the result is only ordinary papillomatosis.[4] This developed often on the back of the neck where the ears had rested and the skin become hairless and somewhat hyperkeratotic. The greater the ear changes at the time of the intravenous injection of virus, the more abundant did papillomas tend to be, hot, thickened, hyperkeratotic ears usually developing them in immense numbers as compared with ears little changed though tarred as often. Yet exceptions occurred. (Compare rabbits 8, 13, Chart 7; 4-72, Chart 11.) And even when the skin was markedly changed, and the animal so susceptible that the virus caused a horde of fast growing papillomas, anomalous tumors often remained completely absent (Fig. 15). Preliminary cytological changes of peculiar sort were obviously essential to their occurrence, changes always focal or punctate, a fact plainly evident no matter how abundant the virus infection. Needless to say the opportunity for the virus to reach cells changed in the appropriate way varied directly with the amount localizing in the ears; and this could be judged from the number of gray papillomas. On scrutinizing the charts it will be seen that, other things being equal, the more abundant these papillomas, the greater was the incidence of anomalous growths, with some exceptions (e.g. rabbit 4-72, Chart 11). Usually the virus caused many papillomas for every anomalous tumor; but again there were exceptions (rabbit 15, Experiment 1). When a dilute inoculum was employed the growths induced were relatively few (Chart 7, Experiment 4), and they appeared late and

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[4] The virus will localize and cause papillomas in skin tarred but once, as Dr. R. J. Parsons has noted.

enlarged slowly, as on scarified normal skin. This was to be expected since the number of cell-virus associations formed at time of infection and producing a growth has much to do with how soon it becomes visible and its rate of enlargement.

Influence of the Virus Strain

Papillomas induced with the virus in scarified normal skin often become carcinomatous after proliferating for some months (8), and the greater the pathogenicity of the inoculum,—as evidenced in the papillomas,—and the higher its titre, the sooner and oftener does the malignant change occur (10). Because of this relationship it seemed well to employ notably pathogenic materials in the present work, and this was done except in the following test.

Experiment 8.—The ears of 31 rabbits tarred 57 days were stripped and charted, tarred again immediately, and 15 of them were injected on the 59th day with 15 cc. of Berkefeld filtrate of a 1.5 per cent extract of the pooled, glycerolated papillomas of 7 cottontails. 12 comparable individuals were reserved as controls, and 4 others had the virus tattooed directly into two strips cleaned of tar along the inside of each ear. This was reapplied to all 2 days later and twice weekly for the following 21 days.

To learn whether the virus would localize in skin inflamed by trauma, the sides of the animals destined for intravenous inoculation were tattooed with sterile needles over an area 2 to 3 cm. across, 3 to 4 hours beforehand. A marked edema developed extending for several centimeters around the traumatized area, which was swollen, ruddy, and ecchymotic by the time of the injection. Within 15 seconds afterwards a similar tattooing with sterile needles was done just outside the swelling, and then the inoculum was tattooed into the other side.

No growths ever appeared at either site traumatized with sterile needles, but the usual crowd of confluent and semiconfluent papillomas slowly formed where the inoculum had been tattooed into the side and somewhat sooner on the tattooed ears. None became malignant during several months observation. More papillomas arose on the ears of the injected rabbits than in Experiment 4, owing doubtless to the larger inoculum, yet in general they appeared only after 4 to 6 weeks, although the tarring had caused pronounced skin changes in many instances, with "warts" in some. In one animal especially susceptible to the virus, some of the latter started to enlarge rapidly when the virus papillomas appeared and later manifested malignancy; but in no other rabbits did cancer appear.

The relatively inactive virus material gave rise to few anomalous tumors, although localizing abundantly in the much changed ears. A constant lookout has been kept for signs of qualitative differences

in the virus materials, as expressed in growths produced. None was encountered in Experiment 6, nor any since. The observation that the sooty papillomas caused by some inocula remained discrete, no matter how close-packed, whereas those due to others soon coalesced, would seem referable merely to differences in proliferative vigor.

The Manifold Effects of Tarring

Without the preliminary cell changes induced by tarring no anomalous tumors would have been got. This constitutes the most significant effect of tar, while it determines the outcome in other important respects as well.

(a) *Virus Localization as Determined by Tarring.*—Several tests were made to learn about the conditions determining localization of the virus. Tattooing the skin while virus circulated (Experiments 4 and 8) did not result in papillomas; but one can assume that blood clots in the puncture wounds held it fast. It is known to localize sometimes at points of actual or presumptive trauma (1) as also in epidermis proliferating in response to Scharlach R (11). In an injected rabbit of the present work papillomas appeared over a subcutaneous abscess. Yet localization in inflamed tissue is far from a regular event as Experiment 8 attests, and also Experiment 9.

Experiment 9.—A strong solution of barium sulfide in water was swabbed on the ears of 3 rabbits to produce acute inflammation. Next day, when this was at its height and the ears much swollen, 15 cc. of the highly pathogenic 0.5 per cent virus fluid of Experiment 4 was injected into a leg vein, 15 seconds later a spot on the side was tattooed with sterile needles, and then the inoculum was tattooed into the other side. Vigorous growths appeared here, but none where sterile needles had been used. Almost no papillomas developed on the ears, 2 in 2 individuals and 6 in the third.

Repeated tarring causes the cutaneous vessels to become highly pathological (12). Their state should provide larger opportunities for the virus to leave the circulation than the changes incident to acute inflammation, which might be favorable only while exudation was taking place.[4] Burrows (13) has discussed the complexities incident to localization of the viruses as a class.

The greater the changes produced by tarring, the more frequent and

profuse, generally speaking, was the localization of the virus. Most of it disappeared from the blood within half an hour (Experiment 5) unless an enormous quantity was introduced, when much was still present after an hour (undescribed tourniquet tests). Our practice was to strip the ears a day or two before the intravenous injection, and sometimes tar was not reapplied until afterwards, or never again. Even within 24 hours their condition had greatly bettered. Under such circumstances less virus should have localized in them than if their pathological state had been maintained; and certainly they developed fewer growths (Experiment 4, Charts 8 and 9). But another factor has to be considered in this relation:—

(b) *The Influence of Tarring to Elicit Growths of Virus Causation.*— Tarring will enable papilloma virus already ensconced in the tissue to produce growths when otherwise it would not do so.

Experiment 10.—Both ears of 6 normal domestic rabbits were infiltrated with 10 cc. each of virus-containing fluid by the method of Experiment 7, with a tourniquet to prevent its escape, which was left on half an hour. 3 days later tarring was begun of the inside of one ear, and repeated 5 times (twice weekly). A multitude of ordinary virus papillomas, sooty and pink, soon arose on the tarred ear, whereas its fellow remained devoid of them during many weeks of observation, save where injury had been inflicted during infiltration with the virus, or hyperkeratosis had been induced by transferred tar (Fig. 21).

In a second experiment 7 days were let elapse before the first of 3 tarrings, and similar results were obtained (Fig. 22). Tests are now in progress to determine how long the virus can lie latent. It is known that under the influence of tarring papillomas which have vanished may reappear (14).

(c) *The Influence of Tarring on Established Growths.*—In several experiments papillomas due to virus that had localized out of the blood appeared on the tarred ears days before any arose where the inoculum had been tattooed into the side; and generally the ear growths enlarged much the more rapidly. These findings,—especially remarkable because of the virus dilution entailed by intravenous inoculation,—must be laid to the existence of highly favorable conditions in the tarred skin. For virus-induced papillomas do not fare notably well on normal ears. The effect of tarring to stimulate both established tar "warts" and virus papillomas has been remarked upon in Paper I. It had still more pronounced effects upon the anomalous

tumors.   Those described in Paper I were tarred during the first weeks of their development.   The photographs show how greatly they differed from the untarred tumors of the present work.   The anomalous growths ulcerate much sooner when tarred, many doing so that are essentially benign, while not a few of the malignant ones invade the adjacent tissues from the first and destroy and eat deep, instead of building up into mounds, discs, or cones.   Tarring notably hastens anaplasia, often rendering this complete.

The morphology and behavior of ordinary virus-induced papillomas are known to be greatly influenced by intercurrent factors (6), and it is reasonable to assume that continued tarring will sometimes cause benign anomalous tumors to progress when otherwise they might not do so.   However this may be, it is certain that some of the anomalous carcinomas reverted to a more orderly though still malignant state after tarring was left off.   This happened with one of the biopsied carcinomas of rabbit 15, Experiment 1 (Figs. 23 and 24); and the conclusion seems warranted that its early anaplastic condition was due to tarring.   Manifestations of factitious malignancy by virus papillomas have been reported in a previous paper (6), and tar papillomas often exhibit the phenomenon.   None of the malignant anomalous tumors of the present work became benign later, yet instances of the sort may be expected.

Do the Anomalous Tumors Derive from Virus Papillomas?

The conditions in tarred skin are so favorable to growth, and tarring so pronouncedly influences morphology, that the question arises of whether the anomalous cancers may not have resulted, one and all, from secondary changes in ordinary, virus-induced papillomas.   On this assumption the progression to cancer,—which requires months in the case of papillomas induced on normal skin,—would be telescoped into a few days.   There is the more reason to consider the possibility because disturbing influences of many sorts will hasten secondary malignancy in virus papillomas.   Tarring might very well be especially potent in this respect.

To settle the point we have followed the course of thousands of ordinary virus papillomas occurring on tarred ears, and have scrutinized a large number microscopically.   Contrary to expectation they retained their initial, benign character throughout the 2 to 5 months of the experiment, doing so even when next anaplastic carcinomas.   In one animal only, tarred for weeks after injection

and retained unusually long (140 days), were cancers found at autopsy in the confluent, fungoid mass on the ears, which might have derived from papillomas secondarily; and in this case secondary carcinomatosis was beginning in the untarred tattoo growth on the side.

It can be stated categorically of the anomalous tumors as a group that they did not derive from ordinary virus papillomas. No proof was obtained that their number can be increased by tarring after the virus has localized in the ears. The procedure does not hasten notably the occurrence of secondary carcinomatosis. Nevertheless it is questionable whether the influence of late tarring to increase the effective localizations of the virus and to stimulate proliferation of the resulting growths will account wholly for the differences manifest in Charts 8 and 9.

The cancers deriving secondarily from ordinary virus papillomas often alter rapidly, changing within a few weeks from convoluted or cystic malignant papillomas to disorderly growths in which papillomatous features can still be recognized, and then to keratinizing squamous cell carcinomas which may finally reach a wholly anaplastic, helter-skelter state. In other instances they retain their initial form even in metastases (8), or hold to one assumed secondarily. The malignant anomalous tumors behave in all these ways,—as do carcinomas of man, for that matter.

The Virus Infection of Tar Warts

Not only did the virus evoke new growths of anomalous character, but often it spurred some of the preexisting tar tumors to unwonted activity, and in not a few cases to malignancy (see Paper I and also rabbit 5-07, Chart 11).

The greater the amount of virus localizing in the ears, as attested by new growths, the more frequently did it affect the preexisting tar tumors, causing them to grow with unprecedented rapidity. Not a few became sooty black (rabbits 18 and 19 of Chart 8; Fig. 20), and sooty spots or segments appeared in many (Chart 8, rabbits 19 and 20), sometimes spreading gradually over their entire surface. The development of secondary resistance in certain individuals led to rapid retrogression of the tattoo papillomas and of the majority of those induced on the ears. At the same time most of the tar tumors stimulated by the virus, and in some instances rendered melanotic, also disappeared (rabbit 18

of Chart 8 and 26 of Chart 9). The tar tumor of Fig. 20 (rabbit 19 of Chart 8), which had become huge and black under the influence of the virus, dwindled when the papillomas did so, and ended as a connective tissue mound covered with smooth epidermis but retaining a gray hue because of many deep-lying phagocytes crammed with the pigment of its earlier activity. Concurrently a nearby tar wart which had become black through the spread of a sooty patch, became pink again, diminishing to a small, indolent hassock such as it had been at first. In the case of rabbit 26 of Chart 9, resistance to the virus waned later, as shown by a reappearance of the tattoo papillomas, and not a few growths on the ears, both pink and sooty, reappeared concurrently. They had just begun to do so at the time of the last charts reproduced. Direct tracings of the tumors in their relative situation to one another had been made on cellophane when they were dwindling, because previous findings (9) had suggested the possibility that the resistance manifested by the host might be transient. The later tracings demonstrated that some of the growths reappeared.

Of especial interest were those numerous cases in which tar tumors stimulated by the virus remained unpigmented, grew fast, and manifested malignancy. Some that had the morphology of carcinomas became enormous and fungoid, but remained uninvasive (rabbit 20, Chart 7, right ear), while others ulcerated destructively and metastasized early. The gross aspect of such growths was the same as that of the anomalous tumors already described, and histologically many resembled the latter; but some had a hybrid morphology. The findings will be detailed in a succeeding paper. Here we are essentially concerned with those growths which appeared where the tarred skin had been devoid of any neoplastic proliferation. The great majority arose at such situations.

DISCUSSION

The findings show that the carcinogenic effect of the papilloma virus upon tarred skin is but one expression of a larger phenomenon, namely the production of a variety of tumors, all differing from those growths of remarkably uniform character which the virus causes under ordinary circumstances. The major factors which enter into the production of the anomalous tumors as a group will now be briefly considered.

Localization of the virus in the tarred epidermis is essential to the occurrence of the anomalous growths, and so too is some preparatory change of the epidermal cells which become infected with it. The

more abundant the localization of the virus the greater is the chance that it will come into association with appropriately changed cells; and the more potent the inoculum, as evidenced in the ordinary papillomas it induces, and the more susceptible the host to its action, the more likely are anomalous tumors to arise, other things being equal. The nature of the preparatory cell change is not clear. It is much more considerable than that determining an effective localization of the virus out of the blood stream; roughly speaking the skin must have been altered by tarring to nearly the extent required for the evocation of tar tumors in some of the rabbits treated. Yet anomalous tumors have often been elicited in ears which gave no sign of such growths, and which indeed had undergone little change as compared with those of other individuals of the same group, tarred to the same extent, receiving the same inoculum, perhaps carrying many tar warts, yet developing only ordinary virus papillomas.

The epidermal changes which have anomalous tumors as their outcome are always focal, the growths arising as discrete entities; and while the foci may be many and close together, when the preliminary tarring has been long, no indication has been obtained that if it were kept up the alteration of the cells would become generalized. When but little virus localizes, or at least causes few papillomas, the discrete character of the accompanying anomalous tumors can be explained to some extent by a spotty escape of virus from the blood through vascular walls that are irregularly pathological (12); but this will not cover those frequent cases in which the virus infects the epidermis almost everywhere, yet still the anomalous tumors appear as sharply defined, scattered entities amidst the sheet of confluent papillomatous proliferation. No evidence has been obtained of a predilection of the virus for cells changed in the way requisite to the production of anomalous growths. On the contrary, cutting down the amount of the inoculum usually results in a rather more than proportionate reduction in the number of such growths arising.[5] In this relation the fact may be germane that the greater the dilution of virus inoculated into normal skin the less frequently does cancer arise secondarily from the papillomatosis engendered (10).

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[5] Under such circumstances the virus is prone to localize in preexisting tar tumors, as would follow from the especially favorable state of their blood vessels.

Frequently the anomalous tumors appeared with a speed that was incompatible with derivation from a single virus-infected cell. At certain spots assuredly the virus must have reached not one element but many which were ready for the production of such neoplasms; and in some instances their composite character gave proof of a multicentric origin (Figs. 16, 17). It was also evident in some of the ordinary papillomas engendered, namely those gray, raised mounds, a millimeter or more in diameter, which appeared soon after the virus injection and grew rapidly, in distinction from the great majority that came later as minute, slowly enlarging, dark points in the skin. The anomalous tumors usually arose even before the most active of ordinary papillomas, however, and for some time exceeded them in rate of growth. The tendency of the anomalous tumors to become broad-based by extension into the adjacent tissue soon after they arose must have contributed to this early superiority. Once the virus papillomas were well established, however, their relatively immense number in many instances, and their extraordinary proliferative energy resulted in masses of tissue which usually occupied much more of the ears than did the cancers, though these eventually dominated through the destruction that they wrought.

The possibility has been kept in mind throughout the work that qualitative differences in the pathogenic potentialities of individual virus entities might exist, and find expression in tumor differences. But no supporting evidence for the supposition has been found, nor evidence of any qualitative difference in the virus strains. It is conceivable, nevertheless, as accounting for the diversity of the anomalous tumors, that the condition to which the epidermal cells had been brought by tarring was such as to suit them to the disclosure of hidden qualitative differences in the individual virus entities, much as an ultrasensitive photographic plate is suited to bring out differences in rays of light that are not apparent under ordinary conditions; but this assumption seems gratuitous.

Tarring the anomalous tumors stimulated their growth markedly and also furthered the anaplasia of such of them as were malignant. Yet contrary to expectation based on these facts, tarring failed to hasten in any significant degree the secondary change of virus papil-

lomas into carcinomas. It is conceivable, indeed probable, that amongst the anomalous growths there were some which verged upon malignancy when they first appeared and were pushed over into it by the stimulation of the later tarring. No such growths came to attention though. As a group the anomalous tumors manifested their distinctive peculiarities from the first, and their number was not increased later by a secondary conversion of ordinary virus papillomas into carcinomas.

The collateral effects of tarring will explain why it evokes tumors sooner than does the potent "carcinogenic" principle, 3:4 benzpyrene, which can be extracted from it. It was chosen for our work, in preference to any of the pure "carcinogens," both because of its effectiveness in this respect and because it brings about marked general changes in the ears, a state of affairs which seemed likely to favor localization of virus out of the blood stream. Lacassagne and Nyka (15) utilizing our general method, have now successfully employed benzpyrene to obtain anomalous tumors with the Shope virus. Their protocols show that the applications to the skin prior to intravenous injection of the virus had extended over many months; yet it does not follow that a shorter period would not have proved effective.

The Shope virus exercises a strict, formative influence upon the growths it engenders in scarified normal skin, these being remarkably uniform in character, benign papillomas of a single type. In tarred skin on the other hand it gives rise to a variety of benign tumors, all with papillomatous features (Fig. 25). The carcinomas that it causes under such circumstances range from the organoid to the anaplastic, and resemble in this respect those deriving secondarily from ordinary virus papillomas, though they show a somewhat greater diversity. All in all the morphological spread of the anomalous tumors is wider, its individual expressions more various, than those exhibited by Shope papillomas and the cancers arising out of them.

SUMMARY

A considerable variety of tumors, both benign and malignant, result from the localization of the rabbit papilloma virus in skin which has

been prepared by repeated tarrings. They appear only in individuals highly susceptible to the action of the virus, and are more likely to be engendered by highly pathogenic inocula. No evidence has been found that differences in the potentialities of the virus entities are responsible for the diversity of the growths. This is referable to changes in the epidermal cells; and much more preliminary tarring is required to produce these changes than suffices to cause localization of the virus out of the blood stream with a resulting papillomatosis of the ordinary sort. The character of the individual anomalous tumors depends in some degree upon the extent of the preparatory changes in the cells, malignant growths being more frequent when the epidermis has been tarred for a relatively long period. All are focal or punctate in origin, and they exhibit their peculiar characters from the first, none being due to secondary alterations in ordinary papillomas. Tarring after the virus has localized in the epidermis does not significantly increase their number. They are the outcome of the state of the cells at the time of virus infection.

Tarring exerts important influences in addition to changing the cells in such a way that unusual tumors result from the action of the virus. The procedure is notably effective in determining localization of the virus out of the blood stream; enables it to produce growths when otherwise it would not do so though present in the tarred skin; stimulates the proliferation of the tumors engendered; makes them disorderly and aggressive; and hastens the anaplasia of such of them as are malignant. It has similar effects upon the tar tumors, as will be demonstrated in a subsequent paper.

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All of the microscopic sections were stained with eosin and methylene blue.

PLATE 18

FIG. 1. Mass of non-pigmented papillomatous tissue, originating from virus-infected epithelium after its extension to cover the raw edge of a hole in the ear. In so doing the epithelium had outstripped the melanoblasts, and hence the growth is not gray like the many papillomas induced by the virus elsewhere on the ear. It did not appear until some days after the gray growths, as would follow from the circumstances of its origin, but it enlarged far more rapidly. $\times \frac{2}{3}$.

FIG. 2. Cross-section of a raised rosette of non-pigmented papillomatous proliferation around the recently healed edge of a punch hole in an ear infected with virus. It resulted from the same conditions as the growth of Fig. 1, and was pink, whereas nearly all of the other papillomas covering the outside of the ear,—save those filling the punch hole,—were dark gray. Much new connective tissue went into its formation. $\times 2\frac{3}{4}$.

FIG. 3. Tumors of the two sorts that the virus generally elicits when it localizes in ears which have been tarred long enough for it to cause cancer (rabbit 4-72, Experiment 5, Chart 11). The large growths appeared first and were all tense, subepidermal hemispheres or globes, and bright pink, the dark hue of some of those pictured being due to dried blood from recent abrasions. These were the *anomalous tumors* of the text. On the ear to the left many minute papules can be perceived in addition, some of them black or gray. They represent the earliest stages of ordinary virus papillomatosis. Few growths of either sort are present on the other ear, owing to obstruction of its circulation for 21 minutes after the virus injection. At this time the tarred skin showed but little change, though one minute tar wart had arisen, which disappeared later. The photograph was taken 27 days after the virus injection, with no further tarring. The arrows point to the growths furnishing Figs. 4 and 5. $\times 5/11$.

FIG. 4. Growths from the ear of Fig. 3 (arrow B). They were punched out on the day after the photograph was taken which provided that figure. The larger tumor had been present about 10 days. Its epithelial layer is seen to be irregularly convoluted, and under high power it appeared to be an invasive, squamous cell carcinoma, with cells dying before keratinization was completed. It was pink in the gross and has stained poorly with methylene blue. The small, darker staining, cystic growth looked like a black dot in the gross. It can be seen to have originated from a hyperkeratotic hair follicle, and it represents an early state of ordinary, melanotic, virus-induced papillomatosis. $\times 9\frac{1}{2}$.

FIG. 5. More growths from the same ear, at a further period of their development. The arrow A of Fig. 3 points to the larger one. They were punched out 10 days after this figure was procured, that is to say on the 38th day after virus injection. Both have erupted. The larger growth is an anomalous tumor and resembles the one in Fig. 4. The small, dark growth at the right is an ordinary, keratinizing, melanotic, virus papilloma. Three other papillomas of this sort can be seen, one of them (P) underlying the anomalous tumor,—which had been present about 3 weeks,—and a second situated at its edge (P²). $\times 14\frac{1}{2}$.

PLATE 19

FIG. 6. A higher magnification of the bracketed portions of the large, anomalous growth of Fig. 5 and of the virus papilloma P^2. The large tumor is seen to be invading along its base (arrow). Its cells show little tendency to keratinize, and they stain lightly with methylene blue. The contrast with the adjacent, orderly, dark, keratinizing growth is great. A hyperkeratotic hair follicle separates them. × 95.

FIG. 7. Biopsy specimen procured 14 days after the infiltration of an ear with virus by way of a marginal vein (D.R. 2-33 S, Experiment 7). The preliminary tarring for 28 days had caused moderate hyperkeratosis but no warts. No more tar was applied after the injection, which was followed in the 2nd week by the appearance of numerous growths. They had been present only a few days when the biopsy was done. The growth A was sooty, B and C pink, this last still subepidermal. Microscopically A proved to be a melanotic virus papilloma, whereas B and C were both anomalous tumors. (See Fig. 8.) × 15.

FIG. 8. The adjacent growths A and B of Fig. 7, as seen in another section made of the region bracketed in that figure,—to show the pronounced differences in character and coloration existing at a spot where the two have coalesced. The cells of the anomalous tumor lie in disorder and are actively invasive (arrows). × 172.

PLATE 20

FIG. 9. Another biopsy specimen containing growths of the same sorts. It was procured from the animal of Figs. 7 and 8, 22 days after the infiltration with virus. Much the same histological differences are visible as in the previous instances. The large, globoid, pink, anomalous tumor had been present 2 weeks, whereas the several sooty papillomas (arrows) are just arising. The large growth has become anaplastic and invasive along the base. × 11.

FIG. 10. Ears of D.R. 5-12 (Chart 11, Experiment 5) photographed 34 days after virus injection,—to show a later stage of the condition pictured in Fig. 3, and from the same experiment. The flow of blood to the ear on the left was blocked for 20 minutes from the beginning of the injection. The anomalous tumors, at first subepidermal and globoid, have become fleshy or dry cones, infiltrating discs, or jagged masses. They are much larger than the sooty papillomas, which appeared later in immense number on the ear with unobstructed circulation, and now cover most of its surface. × 5/11.

FIG. 11. Portion of a metastasis in an auricular lymph node of a rabbit not tarred again after the virus injection (D.R. 4-73, Experiment 5; see also Fig. 12). The nodule was discovered on the 76th day, and was removed on the 90th, when it had almost entirely replaced the gland and was 1.3 cm. across. Bits of it implanted in the muscles of the upper legs gave rise to growths (see Fig. 13). × 11.

FIG. 12. Invasion by the metastasis of Fig. 11. In the region pictured the growth is almost wholly anaplastic. A mitosis can be seen. × 250.
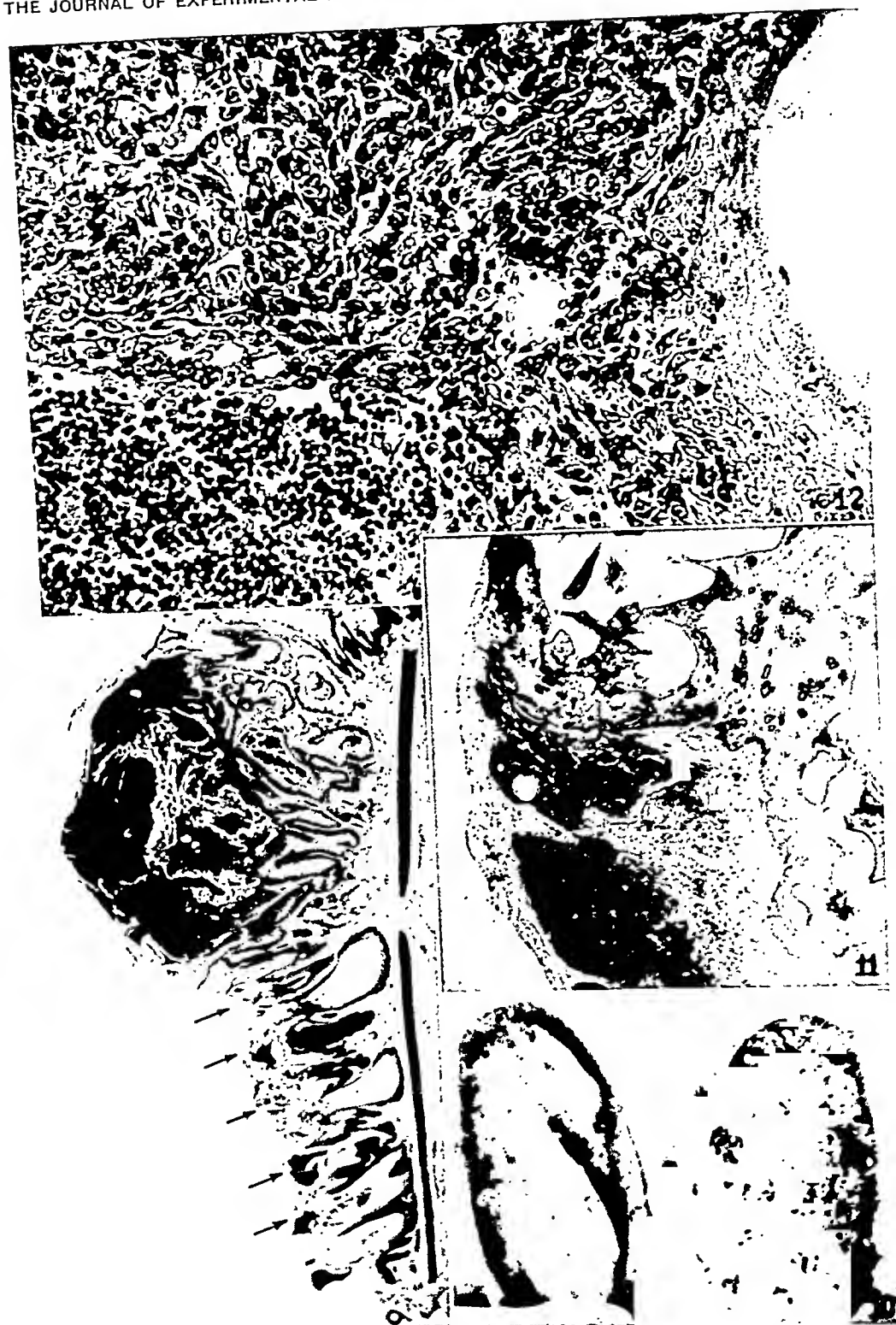
PLATE 20

PLATE 21

FIG. 13. A portion of one of the tumors resulting from implantation in the leg muscles of bits of the growth of Fig. 11. $\times$ 16⅔.

FIG. 14. Two anomalous tumors with the morphology of carcinomas, from the ear of an animal killed 59 days after receiving virus intravenously, with no later tarring (D.R. 4-95 N, Experiment 5, left ear). A single tar wart had been present (Chart 11) at a distant situation. One of the cancers (A) is wholly anaplastic, the other somewhat cystic (B). There is much adjacent virus papillomatosis. $\times$ 6⅔.

FIG. 15. The ears of an animal of Experiment 6 which illustrate the fact that an abundant virus infection of skin much changed by tarring does not necessarily result in anomalous tumors. In the instance presented only one developed amidst the innumerable papillomas. It cannot be seen in the picture. No tar warts were present at the time of injection. $\times$ 5/11.

FIG. 16. A virus-induced, composite growth, from D.R. 2-31 of Experiment 6. It first appeared as a particolored, subepidermal mound which became hemispherical and then onion-shaped. Its central portion, which was gray, is seen to consist of ordinary virus papillomatosis. Its periphery was pink, and where this has been cut through is an area of anomalous growth (C), apparently malignant, joined on to the main mass, while in the region to which the arrow points is a deep, lateral extension from another component of the same sort (C), not visible in its superficial portions in this section. The growth appeared, together with many others, between the 11th and 15th day after the virus injection, and it was punched out on the 21st day. From the first the existence within it of differing neoplastic elements had been plainly visible in the gross. $\times$ 11.
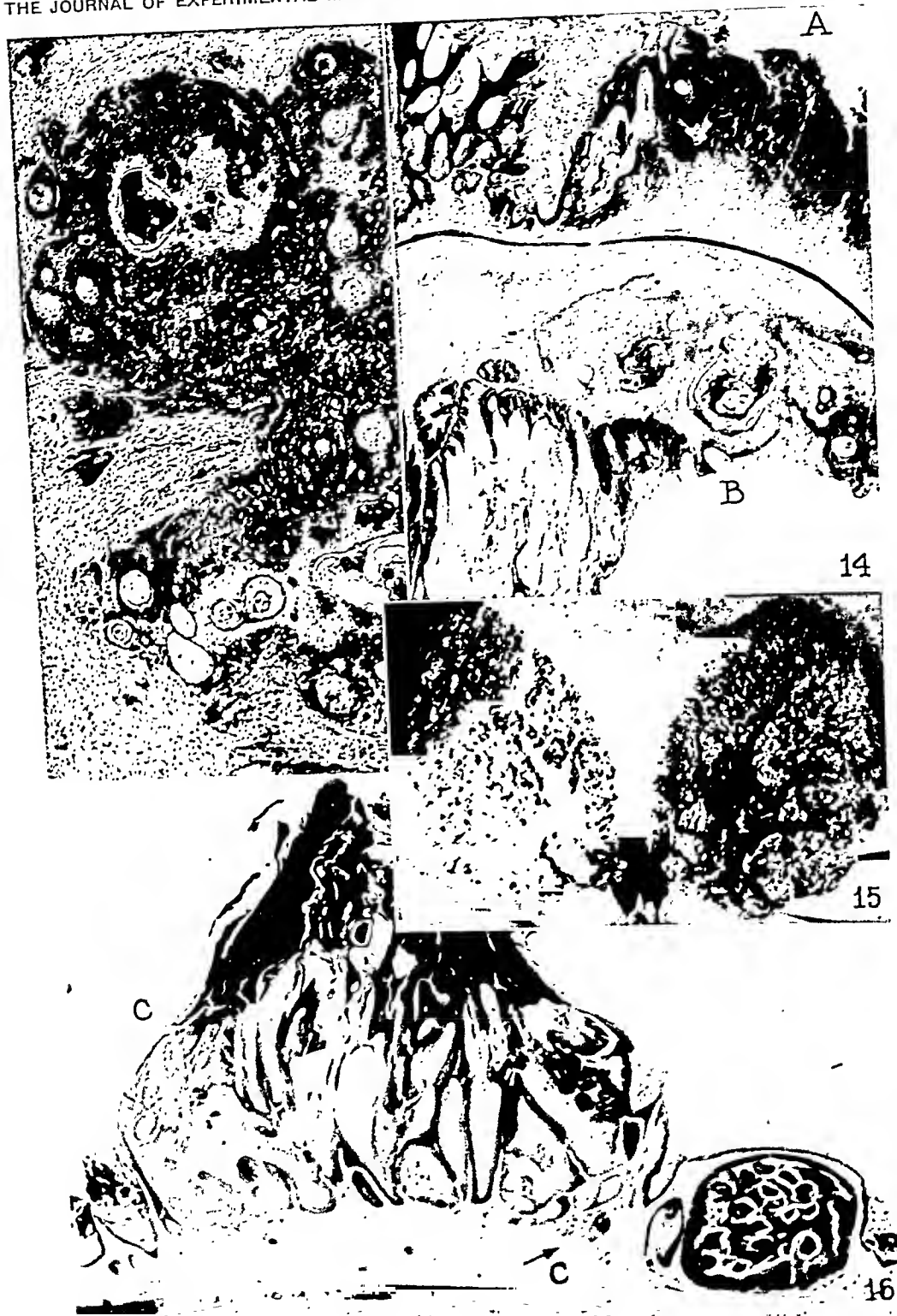
PLATE 22

FIG. 17. A particolored, composite growth from another animal of the same experiment (D.R. 2-56, killed 57 days after virus injection). The ears were largely occupied by virus tumors of various sorts. No tar warts had been present on either at the time of injection, and no later tarring was done. A central region of the growth pictured consists of irregular, cystic tissue. This region was pink in the gross from the first, whereas the remainder of the tumor was dark gray and consisted, as the figure shows, of ordinary virus papillomatosis. × 11.

FIG. 18. Benign and malignant papillomas elicited by the virus on the ears furnishing Fig. 17 (see also Fig. 25). The benign papillomas (P, P, P) stained much the more deeply with methylene blue. The malignant tumor has extended around the edge of the ear. × 6⅗.

FIG. 19. A conical growth with the morphology of a cystic, squamous cell carcinoma, which appeared after the virus had localized in a tarred ear, a fact evidenced by the concurrent appearance of many melanotic papillomas. Parts of some of the latter are included in the picture. No tar warts had been present on either ear and no tarring was done after the virus injection. (D.R. 2-23 S, Experiment 6.) × 10½.

FIG. 20. Secondary melanosis of a tar tumor, as result of virus infection (left ear of Rabbit 19, Chart 8). Both the growths shown had been elicited by the tarring and both were pink prior to injection of the virus, but afterwards one of them became coal black all over and the other developed a dark gray segment, on the side away from the camera. Their growth rate was also markedly increased. Later most of the ordinary virus papillomas elsewhere on the ears disappeared, evidently because of a generalized secondary resistance on the part of the host, and at this time the black growth here shown disappeared entirely, while the pink one (which had now become melanotic everywhere, Chart 8) dwindled, lost its pigmentation, and assumed once again the aspect of an ordinary tar tumor. × 10/11.

FIG. 21. To show that tarring will enable ensconsed virus to produce growths when otherwise this would not happen. Both ears, previously normal, had been directly infiltrated with 10 cc. of virus fluid 3 days prior to the first of 3 tarrings of the ear on the left (Experiment 10). A multitude of papillomas arose on this ear, relatively few on the other, and these mostly where transferred tar rendered the skin hyperkeratotic. The picture was taken 53 days after the virus injection: the differences shown persisted. × 5/11.
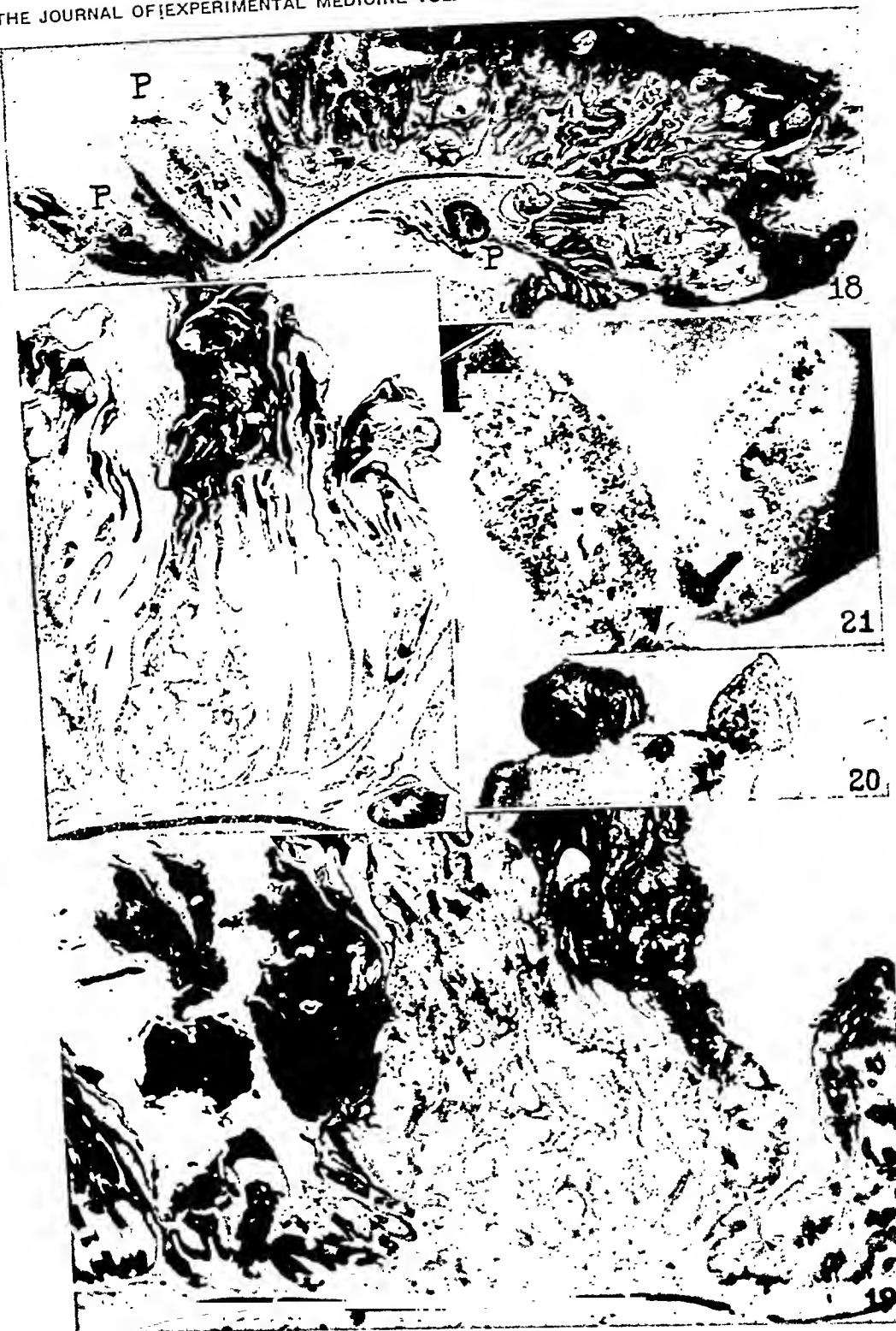
PLATE 22

PLATE 23

FIG. 22. The ears of an animal from another experiment of identical sort save in that the tarring was started 7 days after the infiltration with virus. The picture was taken on the 29th day. No more papillomas developed on the untarred ear before the rabbit was killed, on the 70th day.   × 5/11.

FIGS. 23 and 24.   Portions of an anomalous tumor, as procured by biopsy on the 29th day after virus injection and at autopsy on the 84th day, respectively. Tarring was left off on the 25th day, and thereafter the general state of the ear reverted toward the normal, and the growth became much less anaplastic.   × 52.

FIG. 25. Papillomatous growths of differing patterns, consequent on the action of the virus upon skin prepared by tarring (D.R. 2-56, Experiment 6, an animal devoid of tar tumors at injection and not tarred later).   At P, P, P, P, are ordinary melanotic virus papillomas, stained heavily with methylene blue.   At the edge of the ear, and extending around its edge, is a malignant papilloma of lighter hue, while toward the base are two benign growths of contrasting pattern, one of which has stained fairly well, the other only lightly.   Fig. 17 illustrates a fifth type of neoplastic proliferation occurring in the same animal (see also Figs. 16, 18). $\times 7\frac{3}{16}$.

CELLULAR REACTIONS TO POLYSACCHARIDES FROM TUBERCLE BACILLI AND FROM PNEUMOCOCCI

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PLATE 24

It has been reported previously that tuberculo-polysaccharides have some toxicity for tuberculous guinea pigs (1–3), but our present studies have convinced us that the polysaccharides by themselves are not toxic to normal animals and in comparison with tuberculo-protein are relatively innocuous to tuberculous animals. In regard to cellular reactions, they give rise to one constant phenomenon, namely, a draining of neutrophilic leucocytes from the bone marrow. In our early experiments all the tuberculous guinea pigs that died after having received the polysaccharide had extensive disease and had shown a profound fall in temperature, as in tuberculin shock, after receiving the polysaccharide. It was our opinion at the time that this fall in temperature might have been referable to the presence of traces of protein in the preparations of the polysaccharides since they were not free from nitrogen (3–5). Recent studies by Dr. A. Cournand of the Tuberculosis Service of Bellevue Hospital tend to confirm this view. In 1935 Cournand and Lester (6) reported from a study of clinical tests that tuberculo-polysaccharides induced mixed reactions, immediate and delayed. The polysaccharides used had been prepared by Drs. Heidelberger and Menzel and were not free from nitrogen (7). Drs. Heidelberger and Menzel have since submitted the polysaccharide to tryptic digestion, which abolishes the reactivity of the protein in the precipitin test. With this material Dr. Cournand has found that the delayed reactions with the polysaccharide have been eliminated in large measure. This work will soon be published and we are permitted to cite it.

In the present studies we first submitted normal and tuberculous

guinea pigs to massive intracardiac and intraperitoneal injections of tuberculo-polysaccharide without any deaths that could be ascribed to the material, thus eliminating the possibility of a lethal effect. Second, we followed the immediate effect on the blood cells and the delayed effect, after 24 hours, on the cells of the peritoneal exudates in rabbits following intraperitoneal injections of small amounts of polysaccharides from tubercle bacilli and from pneumococci. Third, we have studied the effect on the bone marrow and on the cells of the blood in rabbits after intraperitoneal injections of small amounts of tuberculo-polysaccharide repeated daily over long periods of time.

Materials and Methods

We are indebted to Dr. R. J. Anderson of Yale University for the polysaccharides from tubercle bacilli. The use of all the materials from tubercle bacilli in these studies has been under plans for cooperative research of the Committee on Research of the National Tuberculosis Association, of which Dr. William Charles White is Chairman. Dr. Anderson gave us three different preparations of polysaccharide. For the first experiment the material used had been separated from the ether-alcohol extract of tubercle bacilli, human strain A-10. Two preparations, also from the human strain A-10, were used for the second experiment. The first was obtained by Dr. Anderson directly from defatted bacilli, that is, from bacilli which had been treated both with ether-alcohol and with chloroform. The second preparation was obtained from analysis of a so called "unfilterable lipid" which had been separated by treating the defatted bacilli with an acid. The chemical studies on these materials will be published by Dr. Anderson in the near future. For the third experiment we used a polysaccharide obtained by Drs. Anderson and Roberts from the ether-alcohol extract of bovine tubercle bacilli (8).

We are indebted to Dr. Michael Heidelberger of the Presbyterian Hospital, New York, for the polysaccharide from pneumococci, Type I, and for the nonspecific, group polysaccharide designated C from pneumococci. The material from the Type I organisms was prepared without heating and was highly purified. It was received in a concentrated solution and was divided into two parts. One part was diluted with distilled water; the other with saline so that 5 cc. contained 10 mg. of the polysaccharide. To Dr. Walther Goebel of the Hospital of The Rockefeller Institute we are indebted for the preparation of the polysaccharide from pneumococci, Type III. It was also prepared without any heating and was highly purified. It was received in saline. The dextrose, trehalose, and the soluble starch were commercial preparations. The soluble starch was given in suspension in distilled water at room temperature. The distilled water used as a diluent came from a metal still and had never been in contact with rubber tubing. It was boiled and then cooled immediately before using.

The blood cells, the peritoneal exudates, and films of omentum were studied both by the supravital technique and after staining with Wright's methylene blue-eosin.

RESULTS

Experiment 1.—Tests for Toxicity of Tuberculo-Polysaccharides. (a) *In Normal Guinea Pigs.*—The polysaccharide used in these experiments was separated from the ether-alcohol extract of human tubercle bacilli, strain A-10, by Dr. Anderson. The material was acid and for some of the injections was adjusted to pH 7 with NaOH. We did not detect differences in the symptoms according to whether the reaction was acid or neutral.

Two normal guinea pigs were given intracardiac injections of large amounts of the material adjusted to pH 7 and dissolved in 2 cc. saline. Guinea pig R 3668[1] received 170 mg. which was 50 mg. per 100 gm. body weight; and guinea pig R 3669 received 380 mg. which was 100 mg. per 100 gm. body weight. Neither animal showed marked symptoms. There was a fall in temperature during the day, about the same amount in the two animals, averaging 3.7°; the temperature had been taken hourly during 3 preceding days and varied about one degree. Four normal guinea pigs which had shown an average variation of 1.5° for the 3 preceding days were given the same amount of saline into the heart and showed an average fall in temperature of 1.5°. Guinea pig R 3668 was twice reinjected with the same polysaccharide but in acid form, 3½ months later. The first of these injections was of 10 mg., given intravenously, and the second of 200 mg., given intraperitoneally. These injections did not cause any symptoms; the following day the animal was sacrificed and showed that there had been a decrease in the number of neutrophilic leucocytes and of mature myelocytes in the bone marrow, and an increase in neutrophils and in macrophages containing neutrophils in the peritoneal exudate.

(b) *In Tuberculous Guinea Pigs.*—The tuberculous guinea pigs, thirty-one in all, which received the injections of the polysaccharide were divided into two groups according to the length of time which had elapsed after the inoculation. They had all been inoculated with 0.01 mg. of strain H-37 in the right groin.

We began the injections of polysaccharide in the first twenty animals 4 weeks after inoculation, when the tuberculin test made with 0.1 mg. of tuberculo-pro-

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[1] These are serial numbers covering the work of the laboratory for a term of years.

tein MA-100 had shown 2 and 3 plus reactions. The remaining eleven were not studied until they had been inoculated for 6 weeks. They had shown 3 and 4 plus tuberculin reactions and some of the group had already died.

Twelve of the tuberculous guinea pigs of the first group received 25 or 30 mg. of the polysaccharide in 2 cc. saline into the heart. Of these, six were given the sugar adjusted to pH 7 and six in the acid form. They all showed the same slight and transient symptoms,—some increase in respiratory rate, moderate chills, and a loss of muscle tone. The temperature fell immediately an average of 1.5° and then rose an average of 3.7°. The range of change in temperature was about the same in all the animals. Two of the animals were found dead the next morning with hemorrhage into the pleura due to a puncture of the tuberculous lung overlying the heart. Eight of the guinea pigs were reinjected into the heart after an interval varying from 2 to 3 weeks with no deaths, except that one of them succumbed immediately from heart block due to puncturing the His bundle.

The remaining eight of the first group received 30 mg. of the polysaccharide intraperitoneally. Six of them were reinjected 2 weeks later. The symptoms were the same as after the intracardiac injections except that there was no loss of muscle tone.

The eleven guinea pigs studied later in the course of their disease were injected intraperitoneally. Three of them received 20 mg. and the rest of them 50 mg. The symptoms were not more marked than in the other group. The temperature, instead of falling, as after the intracardiac injections, rose an average of 3.5°. One of this group died during the following night due to a rupture of the spleen from a needle puncture of the enormously enlarged organ.

In summary, thirty-one tuberculous guinea pigs received injections of from 20 to 50 mg. of the polysaccharide either into the heart or into the peritoneal cavity. Fourteen of the number were reinjected 2 or 3 weeks later with the same amounts. There was no increase in symptoms on reinjection, so there was no sign of any sensitization. After these forty-five injections there were only four deaths, all of them clearly accidental, two from puncture of the lung, one from puncture of the spleen, and one from heart block. These results indicate that the polysaccharides used cannot be considered as having lethal power for tuberculous guinea pigs.

Experiment 2.—Peritoneal Exudates and Blood Cells of Normal Rabbits Receiving Bacterial Polysaccharides.—It has long been known that a wide range of materials,—bacterial proteins, peptones, and various salts,—injected parenterally, call neutrophilic leucocytes from the vessels into the tissues and bring about characteristic changes in

the proportions of the white blood cells. Beard and Beard (9), who counted the blood cells as soon as 10 minutes after intraperitoneal injection of saline, showed that the fall in the number of circulating white blood cells begins at once and involves granulocytes, lymphocytes, and monocytes. This leucopenia is followed by a leucocytosis during the next few hours, which is due to a rise in neutrophilic leucocytes, since both monocytes and lymphocytes continue to fall. The lymphocytes are in the last group of the white blood cells to return to their original level (Sabin *et al.*, 3).

We have made further studies of these phenomena, analyzing the qualitative changes in the neutrophilic leucocytes in the blood stream and following in the peritoneal exudates the reactions that are cor-
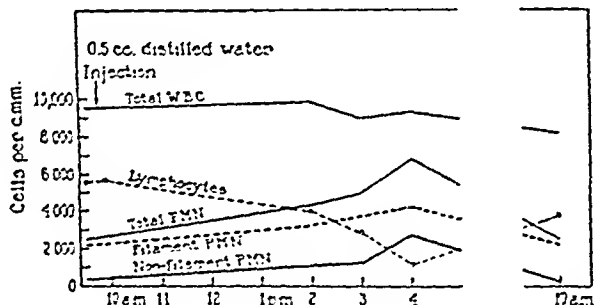
CHART 1. Rabbit R 6043.

related with these changes. We have used five kinds of bacterial polysaccharides, three from pneumococci and two from tubercle bacilli, in comparison with two simple sugars, dextrose and trehalose, as well as soluble starch given in suspension, and certain diluents,— distilled water, saline, and Tyrode solution.

The amount of the sugars injected has been 10 mg. in every instance; the injections were all made intraperitoneally. In the earlier experiments we used 5 cc. of saline or distilled water as the diluent; later we found it better to use a small amount of distilled water, namely, 0.5 cc., since this amount gave the least reaction as a control. It would have been better had this diluent been used throughout the experiment, but it was not possible to repeat all of the injections and the results are still sufficient to show that the more complex bacterial polysaccharides, in comparison with saline and simple sugars, give rise to reactions that are greater in degree though the same in kind.

The effects of these injections were followed both in the peritoneum and in the blood stream.   The studies of the blood cells are illustrated in six graphs, two of which show the effect of the diluents,—distilled water (0.5 cc.), Chart 1, and
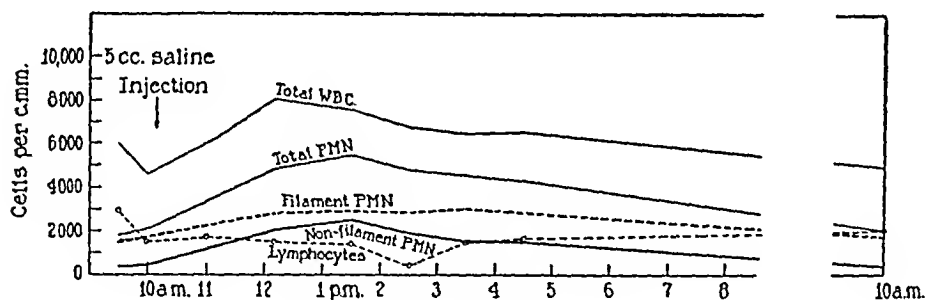
CHART 2. Rabbit R 6257.
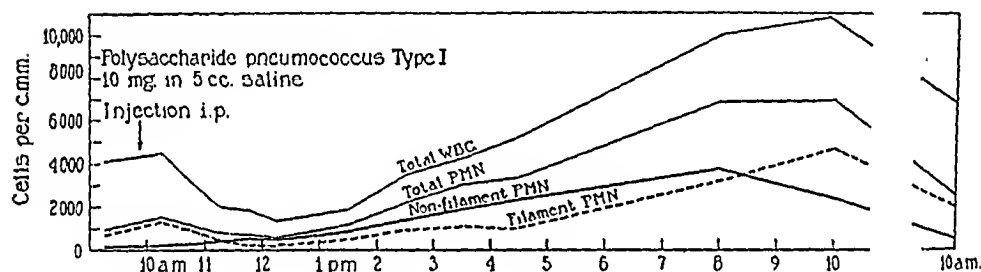
CHART 3. Rabbit R 6113.
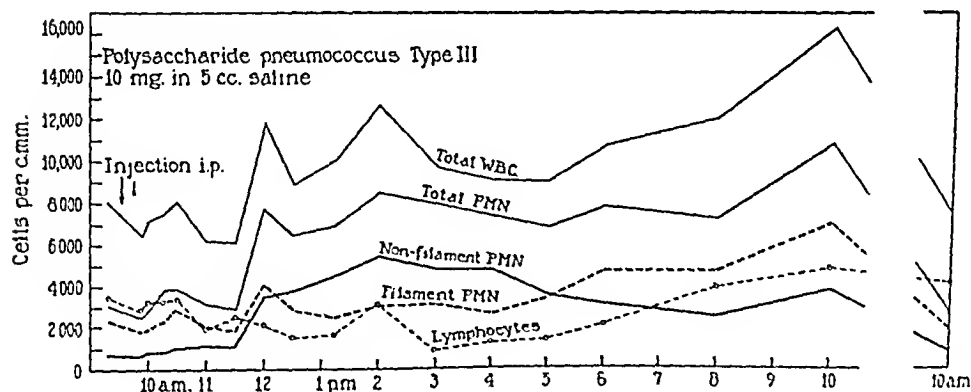
CHART 4. Rabbit R 6119.

saline (5 cc.), Chart 2, while the effects of four different bacterial polysaccharides are illustrated as given in each of these diluents (Charts 3 to 6).   The data used for these graphs were from the group recorded in Table I.   The experimental

procedure was to obtain a base-line of the blood cells of the animal for a few days preceding the injections. These records are not included in the graphs since they were like the count made just before the injections. The counts of the total number of white blood cells per c.mm. were an average from two pipettes; the differential counts recorded in the graphs were all made from fixed films in order to determine the shift to the left (Arneth-Schilling) in the neutrophilic leucocytes.[2]
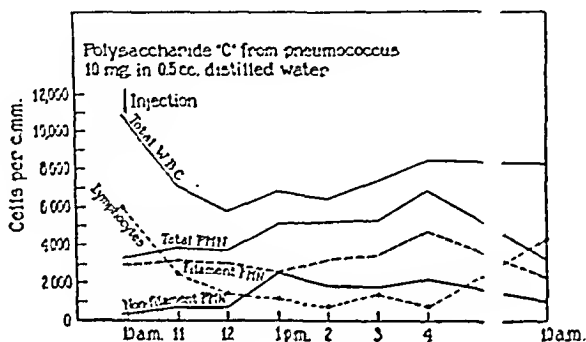
CHART 5. Rabbit R 6340.
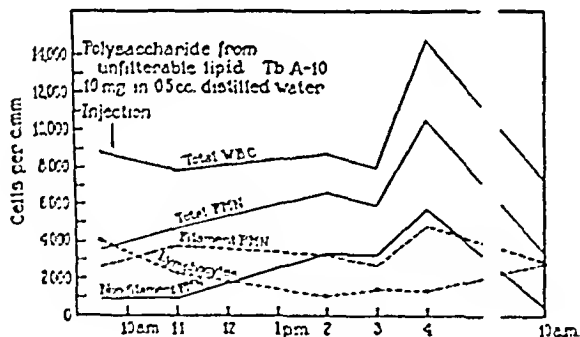
CHART 6. Rabbit R 6125.

Our studies have shown that this shift to the left in the neutrophils of the blood stream is the most accurate measure of the amount of reaction to the peritoneal injections. We made a modified Schilling count, consistently dividing the neutrophils into two groups, those

[2] The term neutrophilic leucocyte is used for the pseudo-eosinophil or heterophil of rabbits' blood since this cell corresponds functionally to the neutrophil of human blood.

with nuclei which showed no filaments whatever, generally conceded to be young forms from the bone marrow, shown in a solid line on the graphs, and the more mature forms whose nuclei do show filaments, indicated by broken lines on the graphs. Differential counts of the

TABLE I

Fall in Lymphocytes in the Blood Stream during 10 to 12 Hours after Intraperitoneal Injections

Number of rabbits	Materials injected	Average number of lymphocytes per c.mm. of blood before the injections	Average of the lowest number of lympho-cytes per c.mm. of blood after the injections	Fall in lympho-cytes
				per cent
6	Distilled H_2O, 0.5 cc.	3327	1532	54
2	Saline, 5 cc.	2050	458	78
2	Dextrose in 5 cc. saline	2131	484	77
4	Tyrode 2–5 cc. 2–0.5 cc.	2584	1052	59
2	Trehalose in 0.5 cc. distilled H_2O	3033	1361	55
2	Soluble starch in 0.5 cc. distilled H_2O	3979	1089	73
Average...				66
2	Polysaccharide, pneumococcus I in 5 cc. saline	1846	605	67
2	Polysaccharide, pneumococcus III in 5 cc. saline	2622	1030	61
2	Polysaccharide, C pneumococci in 0.5 cc. distilled H_2O	5007	834	83
2	Polysaccharide, unfilterable lipid tubercle bacilli in 0.5 cc. distilled H_2O	3384	1274	62
1	Polysaccharide, defatted tubercle bacilli in 5 cc. saline	1118	324	71
Average...				69

cells were made just before the injections and then within 15 to 30 minutes afterward, followed by counts at hourly intervals throughout the day. In the case of the rabbits receiving distilled water, we counted the blood cells only in the afternoon (Chart 1). As the graphs

show, there was a slight immediate drop of the blood cells, followed by a rise which was due to both forms of neutrophilic leucocytes. The proportional rise of the immature to the mature forms was considered as the index of the amount of the reaction. The immature forms, the neutrophils whose nuclei showed no filaments, did not rise above the mature leucocytes with either diluent, or with either of the simple sugars, dextrose or trehalose. These immature forms rose above the mature neutrophils in both of the animals which received the soluble starch and after almost all of the injections with the bacterial polysaccharides. There were two exceptions, namely, one of the two rabbits which received the C polysaccharide from pneumococci, as shown in Chart 5, where the numbers became equal, and one of the animals that received the polysaccharide from the unfilterable lipid of tubercle bacilli. Thus, in general there was a greater reaction of the neutrophils after the bacterial polysaccharides than after the simple sugars, as will be evident also in the studies of the cells of the peritoneal exudates.

In Table I are shown the changes in the lymphocytes following these injections. These records are also taken from the differential counts made from fixed films. After every injection, whether of diluent or sugars, there was a fall of the lymphocytes in the blood stream. The prolonged fall of the lymphocytes and their slow recovery are also illustrated on the graphs, except that the line was not included on Chart 3 where the other lines were so close together. In Table I are presented the total number of lymphocytes per c. mm. before and the lowest number of lymphocytes after the injections. As the graphs show, the lowest number of lymphocytes was reached 4 to 6 hours after the injections. The amount of the fall of lymphocytes in the blood shown was practically the same for each animal and therefore was the same for the two groups, those that received only the diluents and simple sugars (fall of 66 per cent) as contrasted with those that received the bacterial polysaccharides (fall of 69 per cent).

In Table II is shown a study of the cells of the peritoneal exudates arranged approximately in accordance with the percentage of free neutrophils. It will be noted that in the data from the effects of the polysaccharide from the unfilterable lipid, the cells from two animals (R 6125 and R 6126) are recorded separately. This was because

TABLE II

*Cells of Peritoneal Exudates**

Rabbit No.	Materials injected	Amounts and diluents	Total number of cells per c.mm.	PMN	Lymphocytes	Monocytes		Amount of cellular reaction
						Normal	With phagocytized PMN	
				per cent	per cent	per cent	per cent	
R 6285 R 6286	None	—	1700	0	5	92	—	
R 6038 R 6039 R 6040 R 6041 R 6042 R 6043	Distilled water	0.5 cc.	5100	14	12	61	12	Slight
R 6114 R 6117 R 6260 R 6261	Tyrode	2–0.5 cc. 2–5.0 cc.	5200	17	5	57	21	"
R 6186 R 6435 R 6436	Trehalose	10 mg., 2–in 0.5 cc. dist. H_2O 1–in 5 cc. saline	4916	32	1	57	9	"
R 6256 R 6257	Saline	5.0 cc.	6925	34	3	42	21	Moderate
R 6437 R 6438	Soluble starch	10 mg. in 0.5 cc. dist. H_2O	4850+ clots	42	4	36	18	Marked
R 6220 R 6239	Dextrose	10 mg. in 5 cc. dist. H_2O	6800	56	1	31	11	"
R 6113 R 6120	Polysaccharide from pneumococcus Type I	10 mg., 1–in 5 cc. dist. H_2O 1–in 5 cc. saline	7712	50	2	36	11	Moderate

* In these exudates there was an average of 0.2 per cent PME and 0.5 per cent serosal cells which have been omitted from the table.

TABLE II—*Concluded*

Rabbit No.	Materials injected	Amounts and diluents	Total number of cells per c.mm.	PMN	Lymphocytes	Monocytes		Amount of cellular reaction
						Normal	With phago-cytized PMN	
				per cent	*per cent*	*per cent*	*per cent*	
R 6119 R 6264	Polysaccharide from pneumo-coccus Type III	10 mg. in 5 cc. saline	7225+ clots	51	21	17	11	Marked
R 6340 R 6341	Polysaccharide C pneumococ-cus	10 mg. in 0.5 cc. dist. H₂O	Clotted	61	1	16	22	"
R 5664 R 5665 R 6049 R 6111 R 6118	Polysaccharide from defatted tubercle ba-cilli, human strain A-10	10 mg., 2–in 1 cc. saline 3–in 5 cc. saline	5550+ clots	58	·3	29	10	"
R 6029 R 6030 R 6115 R 6116	Polysaccharide from unfilter-able lipid	10 mg., 4–in 5 cc. saline 2–in 0.5 cc. dist. H₂O	7725	81†	1	12	6	"
R 6125 R 6126			Clotted	24†	6	38	30	"

† This was the only instance in which there were marked differences in the differentials with different diluents and so they are recorded separately.

these two differential counts, which were alike, varied from the others in the group. The difference is, however, not so extreme if it be noted that the monocytes containing neutrophils were very high in these two animals. The total number of cells in the peritoneal fluid cannot be obtained accurately after the injections of the sugars because the fluid clots so quickly. The procedure has been to have the pipettes rinsed with a solution of heparin and then to fill them as soon as possible on opening the peritoneal cavity. If only small clots formed, the counts were made to obtain some approximation of the number of cells present. The constant reaction has been an increase in neu-

trophils in the exudate and the phagocytosis of them by monocytes. The phagocytic mononuclear cells (monocytes, clasmatocytes, or macrophages) have been notably in the milk spots of the omentum, to some extent under the serosal lining of the peritoneum, in the sinuses of the retrosternal lymph nodes, and, to a marked degree, in the spleen. A few of the cells with phagocytized neutrophils wandered into the peritoneal exudate. In the last column in Table II is an estimate of the amount of these two reactions, the exudation of neutrophils and their phagocytosis in the omentum, as seen in films and in sections, and in the lymph nodes and spleen. As will be seen in Table II, the least reaction was obtained from distilled water, Tyrode solution, and the simple sugar, trehalose. On the other hand, soluble starch in suspension, dextrose, and all the bacterial polysaccharides except one induced marked reactions.

Two rabbits, R 6342 and R 6343, were studied 1 and 6 hours after the intraperitoneal injection. These animals received 10 mg. of tuberculo-polysaccharide from the unfilterable lipid in 0.5 cc. distilled water. Thus the experiment was like that recorded in Chart 6, in which it will be noted that the greatest fall in neutrophils was at the end of 1 hour and the greatest rise in them at the end of 6 hours after the injection. At the end of 1 hour (R 6343) there was a slight increase in the amount of fluid in the peritoneal cavity; the total number of cells per c.mm. of the exudate was 7450 and the fluid did not clot until it had stood for some time. The differential count showed 98 per cent active motile neutrophils, 1 per cent lymphocytes, and 0.5 per cent monocytes and 0.5 per cent serosal cells. At the end of 6 hours (R 6342) the fluid was scanty and clotted so quickly that the count of 8000 cells must be considered as too low; the differential count showed 94 per cent active motile neutrophils; 1.5 per cent rounded neutrophils, 2 per cent eosinophils, 1 per cent lymphocytes, and 1.5 per cent monocytes. There were no phagocytic cells containing neutrophils in the exudate and none were found in the omentum, in the retrosternal lymph nodes, and none in the spleen.

In contrast to these peritoneal exudates of active neutrophils during the first 6 hours after intraperitoneal injection of a tuberculo-polysaccharide are the late reactions 24 hours after the injections. At this time the exudates are mixed and consist of both neutrophils and monocytes which have phagocytized them. This is true both after the simple materials and after the bacterial polysaccharides. These later reactions are shown in Figs. 1 to 4.

The exudates studied 24 hours after injection showed considerable evidence of damage, or at least of aging of the neutrophils. In the supravital films many of the neutrophils were round and showed neither streaming of granules nor movement of the cells; in fixed films it could be seen that many of them had fragmented and pyknotic nuclei. These points are clear in Fig. 1, which is from a photograph of a fixed film of a peritoneal exudate from a rabbit 24 hours after it had received 10 mg. of a tuberculo-polysaccharide from defatted tubercle bacilli. This photograph also shows monocytes both with and without phagocytized neutrophils, as well as many free neutrophils. Most of the actively phagocytic cells were found not free in the exudates but rather in the milk spots of the omentum, in the sinuses of the retrosternal lymph nodes, and in the spleen. Every animal showed a considerable reaction of the phagocytosis of neutrophils in the spleen. In Fig. 2 is shown a milk spot of the omentum of a rabbit (R 6040) 24 hours after an injection of 0.5 cc. of distilled water. The majority of the cells are monocytes and near the lower margin is a group of monocytes, one with two nuclei and one with two phagocytized neutrophils. In contrast to the small amount of reaction, both of free and phagocytized neutrophils in this photograph, is the reaction shown in Fig. 3, 24 hours after injection of 10 mg. of the C polysaccharide from pneumococci. This is also a milk spot and shows many free and phagocytized neutrophils. The neutrophils which had been phagocytized were not only those with pyknotic nuclei but also leucocytes which, though they were within monocytes, still looked normal. These phagocytized neutrophils were found in various stages of disintegration; in some of the monocytes there were only a few fragments of what seemed to be nuclear debris; in other phagocytic cells, especially after both forms of type-specific polysaccharides from pneumococci, were clumps of debris which we interpret as cytoplasmic. Such a cell is shown in the center of Fig. 4 from a film of omentum of a rabbit (R 6264) which had received 10 mg. of the polysaccharide from Type III pneumococci. This debris is in small clumps, vaguely suggesting platelets; it stained purple in Wright's eosin-methylene blue after fixation with dioxane and methyl alcohol. After this fixative the metachromatic basophilic granules are well preserved and are easily discriminated from this material. Every transition between the cytoplasm of the neutrophils and this granular material can be made out in these omental films. These reactions indicate that the phagocytic mononuclear cells are able to disintegrate neutrophils quickly. Previous observations have shown that the reaction is practically complete in 48 hours. The two upper cells in Fig. 4 are serosal cells, one of which shows clear vacuoles. These correspond to refractile droplets of lipid, as seen in the living cell. They are a constant reaction of irritation in serosal cells.

Experiment 3.—Effect of Daily Injections of Tuberculo-Polysaccharide on the Cells of the Blood and Bone Marrow.—In the third experiment two normal rabbits received daily (except Sundays) intraperitoneal

injections of 10 mg. of tuberculo-polysaccharide obtained from bovine tubercle bacilli (8) in 1 cc. distilled water. Rabbit R 2178 received these injections for a period of 6 weeks; rabbit R 2179 for 6 months. This experiment was planned to ascertain the effect on the bone marrow of repeated injections of this material.

In these animals the blood cells were counted frequently and no effort was made to follow the reactions on the blood cells during the first 12 hours after the injections as in the preceding experiment. The differential counts were made with the supravital technique. During the period of the injections there were no

TABLE III

Means of Blood Cells before and during Period of Injection

Rabbit No.	White blood cells		PMN		Lymphocytes	
	Before injection	During period of injection	Before injection	During period of injection	Before injection	During period of injection
R 2178	10,275	12,864	4815	5708	2734	4926
R 2179	11,300	10,189	7101	5314	2998	3127

TABLE IV

Proportion of Myeloid to Erythroid Cells in the Bone Marrow after Repeated Injections of Tuberculo-Polysaccharide

	Rabbit R 2178	Rabbit R 2179
	per cent	*per cent*
Myeloid cells.	71.2	57.6
Erythroid cells.	17.9	36.4
Primitive cells.	10.9	5.9

important changes in the total number of white blood cells and in the total number of the neutrophils and lymphocytes per c.mm., as shown in Table III. When the animals were sacrificed 24 hours after the last injection, rabbit R 2178 showed some thickening of the omentum and a considerable number of neutrophils in the peritoneal exudate and in the omentum, both free and within monocytes or macrophages. There had also been active phagocytosis of the neutrophils in the spleen and the bone marrow was moderately hyperplastic. Rabbit R 2179, which had received the injections for a longer period, showed so much thickening of the omentum that a film could not be made for supravital study. Both the omentum and the peritoneal lining of the body wall showed layers of large monocytes, some of them containing nuclear debris, probably from phagocytized neu-

trophils. There were also a few giant cells. There were fewer neutrophils either free or phagocytized than in rabbit R 2178. The bone marrow was hyperplastic throughout, with, however, some fat cells remaining; there was well marked extramedullary formation of neutrophilic myelocytes in the spleen. The counts of the cells of the marrow of these animals showed an increased proportion of myeloid to erythroid cells in each instance, as shown in Table IV.

DISCUSSION

The fact that such large amounts of tuberculo-polysaccharides as have been given not only to normal but to tuberculous guinea pigs did not elicit marked symptoms indicates that the harmful effects of these sugars are slight. Perhaps they cannot be considered as entirely non-toxic or innocuous since, as Cournand and Lester (6) have shown, a characteristic skin reaction can be elicited with certain polysaccharide fractions in tuberculous patients.

These studies have shown that polysaccharides from tubercle bacilli and from pneumococci introduced into the peritoneal cavity of rabbits call neutrophils from the circulation in larger numbers than the same amount of normal saline or simple sugars. This phenomenon in general is well known and has been submitted to extensive study both with bacterial products and with many different kinds of salts (3, 9, 10–16). As has been shown, there is an immediate fall in the white blood cells, including neutrophils, monocytes, and lymphocytes (9). The leucopenia becomes maximum in 1 hour, when the neutrophils begin to rise, while monocytes and lymphocytes continue to fall. The lymphocyte is the last of the three cells to rise and does not reach its original level for 24 hours (Sabin et al., 3).

In the present studies it has become clear that the lymphocytes react entirely differently from the neutrophilic leucocytes. There is a prolonged and steady fall in lymphocytes, lasting from 6 to 8 hours; during this period, as well as for 24 hours later, there is no exudation of lymphocytes into the peritoneal cavity. This is clear in the cells of the exudates of the rabbits studied on the day of the injections, where only 1 per cent of lymphocytes were found, as well as in the low percentages of lymphocytes in the exudates 24 hours after injection, as shown in Table II. Also careful studies of the condition of the lymphocytes in the films of blood cells have failed to reveal any signs of degeneration of lymphocytes. From these observations it seems

likely that even the slight irritation of the peritoneum brought about by these injections has retarded the delivery of lymphocytes into the circulation. This hypothesis might be tested by finding the rate of delivery of lymphocytes through the thoracic duct after such intraperitoneal injections.

The study of the reactions of the neutrophilic leucocytes, on the other hand, indicates that two entirely different mechanisms of the bone marrow are brought into play. There is, first, a mechanism for the quick delivery of neutrophils into the blood stream and, second, a building up of the bone marrow after depletion. The first mechanism comes into play about an hour after the injection and acts for from 6 to 10 hours. This is illustrated in the graphs, where the rise in neutrophils has usually lasted for 6 hours but in one instance for 10 hours (Chart 3). During this period no damage to the extravasated neutrophils was detected in peritoneal exudates and no phagocytosis nor digestion of them was found either in the omentum or in the spleen. Only one observation gives any suggestion of a possible chemical stimulus from the cells available during this period. By the 6th hour there is a marked and rapid clotting of the peritoneal exudate. From many observations on these peritoneal exudates we have found that such rapid clotting is correlated with the appearance in the exudates of fragments of the surface films of cells, usually of monocytes. Such cellular debris is shown in the lower edge of Fig. 1. Fragmentation of bits of the cytoplasm from the neutrophils is frequently seen in supravital films of blood cells and may have taken place in the peritoneal exudates in from 6 to 10 hours concomitantly with the increase in clotting time of the fluid. However, it seems to us that the speed with which the delivery of young neutrophils from the marrow to the blood stream takes place suggests rather that the materials injected, salts or sugars, which have attracted the neutrophils from the peripheral vessels into the tissues, when they arrive in the sinuses of the bone marrow, attract the young neutrophils from the marrow into the sinuses. It is well known that in the peripheral vessels the neutrophils move along the inner lining of the endothelium, while in the marrow the young neutrophils lie along the outer surface of the endothelium of the sinuses. Thus the conditions are the same, except that in the periphery the stimulus is

outside the vessels and by chemotaxis draws the cells into the tissues, while in the marrow the stimulus may be within the vessels. The marrow is the only place where there is a supply of neutrophils outside the vessels, so it is in this organ that the new neutrophils are drawn into the vessels. This phenomenon takes place before the disintegration of the extravasated neutrophils can be detected. Generally speaking, it is the reaction during the first 12 hours after the injection of foreign materials into the tissues. It is, of course, true that there may be some peripheral redistribution of cells in the blood vessels, as, for example, from the spleen during these early hours after intraperitoneal injections, but it seems to us that the most likely explanation of the phenomenon is that some of the materials injected reach the marrow before they can be eliminated by the kidneys and serve as the agent for the depletion of the marrow of young leucocytes.

Entirely different from these reactions of the first 12 hours after intraperitoneal injections, are the phenomena to be made out during the next 12 hours. Between 12 and 24 hours there have been not only an aging of the extravasated neutrophils but a marked phagocytosis of them by monocytes. It is our opinion that the neutrophils which have emigrated from the vessels remain in the tissues where, if in not too overwhelming numbers, they are phagocytized and disintegrated within a short time. It was such observations that initiated the study of the action of nucleotides (Doan, Zerfas, Warren, and Ames, 17; Doan, 18) on the bone marrow. It seems likely to us that all of the material of the neutrophils, cytoplasmic as well as nuclear, is broken down by the phagocytic mononuclears and returned to the blood stream. The experiments involving injections of the polysaccharides over weeks and months suggest that this disintegration of extravasated neutrophils is correlated with the building up of the marrow after depletion, that is, with the cell division and the maturation of melocytes. The experiments with repeated injections of small amounts of polysaccharide are an example of a nice adjustment of the marrow by which the disintegration products of the leucocytes withdrawn from the circulation make the marrow just hyperplastic enough to replace them. The result was a normal blood count and a hyperplastic marrow.

Our observations indicate that there are two phases of the reactions

of the bone marrow to intraperitoneal injections of materials that are sufficiently innocuous to reveal a physiological mechanism. There is first a withdrawal of leucocytes from the peripheral vessels and a compensating draining of new neutrophils into the sinuses of the bone marrow. The stimulus for the first and probably for both of them is the material injected. In the periphery the stimulus is outside the vessels; if our theory is correct, in the marrow it is within the lumen of the sinuses. There is second a building up of the marrow through the products of disintegration of the extravasated neutrophils brought about by the phagocytic mononuclear cells. These two phenomena are separated in time. In both instances the forces involved were nicely balanced under the conditions of our experiments.

SUMMARY

1. Purified tuberculo-polysaccharides are relatively innocuous both to normal and to tuberculous guinea pigs.

2. Both tuberculo-polysaccharides and polysaccharides from pneumococci call larger numbers of leucocytes from the blood vessels than do saline and dextrose and trehalose.

3. The mechanisms controlling the delivery of lymphocytes and neutrophils into the blood stream are different.

4. Slight irritation of the peritoneal lining slows the delivery of lymphocytes to the blood stream.

5. There are two phases in the reaction of the bone marrow to intraperitoneal injections. Correlated with the draining of neutrophils from vessels to tissues, owing to the presence of foreign materials in the latter, there is a draining of young neutrophils from the marrow into the sinuses of the marrow as these same materials reach the sinuses. The subsequent disintegration of the neutrophils extravasated into the tissues is correlated with increased myeloid activity in the marrow.

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EXPLANATION OF PLATE 24

FIG. 1. Peritoneal exudate from rabbit R 5665, 24 hours after an intraperitoneal injection of 10 mg. of polysaccharide from defatted tubercle bacilli. Fixed for 2 minutes in dioxane, 30 parts, and methyl alcohol, 70 parts, and stained with Wright-Giemsa. × 1200.

FIG. 2. Film of omentum of rabbit R 6040, 24 hours after an intraperitoneal injection of 0.5 cc. distilled water. Fixed in dioxane, 30 parts, and methyl alcohol, 70 parts, for 3 minutes and then stained with Wright's methylene blue-eosin. × 550.

FIG. 3. Film of omentum of rabbit R 6340, 24 hours after an intraperitoneal injection of 10 mg. of C polysaccharide from pneumococci. Fixation and stain as in Fig. 2. × 1000.

FIG. 4. Film of omentum of rabbit R 6264, 24 hours after an intraperitoneal injection of 10 mg. of a polysaccharide from pneumococci Type III. Fixation and stain as in Fig. 2. × 1000.
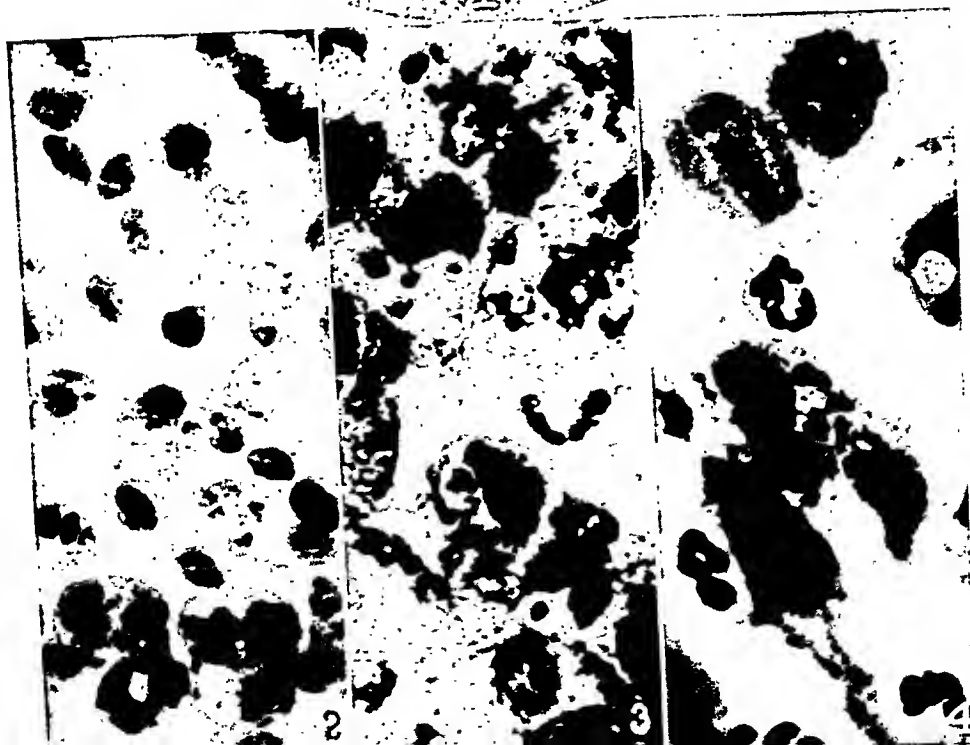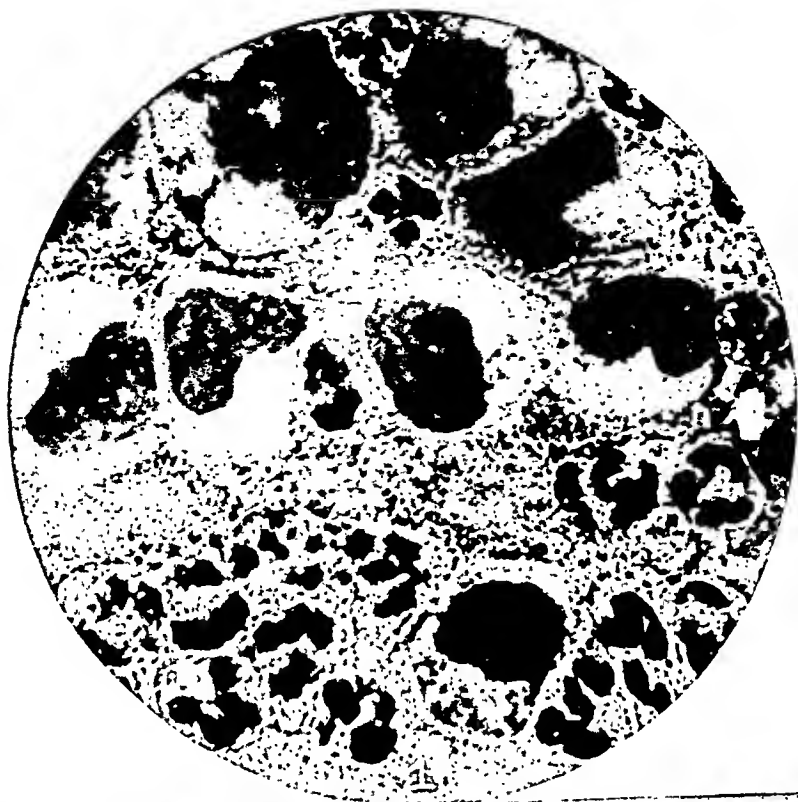
ULTRACENTRIFUGATION STUDIES ON THE ELEMENTARY BODIES OF VACCINE VIRUS

I. General Methods and Determination of Particle Size

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The work of Stanley (1) which indicates the protein character of the etiological agent of the first recognized virus disease, tobacco mosaic, has added to the speculation regarding the nature of the elementary bodies associated with certain of the larger viruses. Elementary bodies of vaccinia either represent the virus itself or else are so intimately associated with it as to be inseparable (2–4). These bodies are similar to bacteria and protoplasm in their content of protein, fat, and ash (5); nevertheless, they give no evidence of respiratory activity *in vitro* (6). It was considered of interest to obtain further evidence regarding the size and density of elementary bodies of vaccinia and to ascertain to what extent they respond to osmotic influences.

In the present experiments the behavior of elementary bodies was studied by photographic methods. Sedimenting boundaries of virus particles were obtained by means of the air-driven centrifuge of Bauer and Pickels (7), and the light-absorption method of Svedberg (8) was employed for the measurement of sedimentation rates. Changes in the physical nature of the elementary bodies could thus be followed by the reflected changes in sedimentation rate. In our experiments, as well as in those of previous workers (9–12) who have studied the size or density of vaccine virus by centrifugal methods, the assumption is made that elementary bodies are spherical or approximately spherical. The ultraviolet light photomicrographs of Barnard (13) would indicate that this assumption is justified.

Materials and Methods

Preparation of Diluents for Virus Suspensions.—The pH values of the suspending media were determined generally by the glass electrode method. In some instances, however, colorimetric indicators were employed. Disodium phosphate-citric acid buffers were used to bring all solutions to the desired pH, usually 7.2; in two instances runs were made with dilute buffer solutions having values of 6.2 and 8.0. Because normal rabbit serum, when present in small amounts, is known to increase the stability of elementary body suspensions (14), 2 per cent by volume was used in many of the test solutions, and a 10 per cent concentration was used in one experiment.

Viscosities were measured with an Ostwald viscometer which had been carefully calibrated. The viscosity value determined for dilute buffer solutions, with and without 2 per cent rabbit serum, was the same as that for water to a close approximation. Whenever the value was appreciably different, a correction was made in computing the sedimentation rate in accordance with the equations given below. The densities of the sucrose, glycerol, and urea (all of c.p. grade) solutions were carefully adjusted to the desired values by the ordinary pycnometric methods. The densities of all other buffer solutions with or without rabbit serum were found to be close enough to 1.00 gm. per cc. to make no density corrections necessary.

Preparation of Virus Suspensions.—The CL strain of dermal vaccine virus which has been carried in rabbits by dermal inoculation of elementary bodies for the past 4 years in Dr. Rivers' laboratory at The Rockefeller Institute was used throughout the experiments. Elementary body suspensions were prepared from infectious rabbit dermal pulp by the differential centrifugation technique of Craigie (2). Washed virus particles obtained from groups of four rabbits were taken up in 100 cc. of a 1/50 dilution of standard disodium phosphate-citric acid buffer, pH 7.2, and stored at 5°C. in flasks containing small amounts of ethyl ether. Preliminary experiments indicated that elementary body suspensions prepared according to routine, *i.e.*, three washings in a Swedish "angle centrifuge" followed by horizontal centrifugation at 2500 R.P.M. for 1 hour to remove clumps, gave as consistent results as more thoroughly washed suspensions.

Elementary bodies were generally prepared for ultracentrifugation in the following manner: 4.5 cc. of stock virus suspension were centrifuged for 1 hour in the Swedish angle machine in a flat pyrex tube with an inside width of 4 mm. Supernatant fluid was poured off and the tube was inverted on blotting paper for 1 minute. While the tube was still held in the inverted position, residual drops of fluid were removed by touching them with bits of absorbent paper. The sedimented elementary bodies, which had a volume of about 0.01 cc., were spread about the bottom of the tube with a glass rod; 3 cc. of the test solution were added drop by drop at first, with constant stirring, and then the suspension was vigorously shaken. The suspension, still in the flat tube, was centrifuged

horizontally in a properly cushioned cup for 5 minutes at a speed of 1000 R.P.M. The supernatant elementary body suspension was poured into a test tube and a portion of it used to fill the ultracentrifuge cell. When this procedure was followed, a minimum of 12 to 15 minutes elapsed between the first contact of the elementary bodies with the test solution and the loading of the ultracentrifuge cell. In the latter part of the work it was found desirable to obtain the sedimentation rate immediately after resuspension of the virus particles. The initial centrifugation of the virus in the test solution was then omitted. It was thus possible to load the ultracentrifuge and bring it to proper speed for the first photograph within 5 minutes after the virus had come into contact with the test solution.

Certain stock suspensions of elementary bodies exhibited a varying amount of autoagglutination; slight settling of agglutinated particles occurred in the storage flasks after a week or two at 5°C. This phenomenon was unpredictable. Stock suspensions which were unstable in this respect were sometimes used in the early experiments and often failed on ultracentrifugation to display boundaries sufficiently well defined for accurate interpretation. It was found expedient to use only stock virus which was stable after storage over a period of 2 weeks to a month.

Virus preparations were stained by the Morosow technique (15) and examined microscopically in order to demonstrate the purity and monodispersion of the suspensions. Dark-field observations were also made of unstained preparations.

Certain terms employed in the discussion of experiments should, perhaps, be defined at the outset. Stock suspensions or lots of elementary bodies refer to the pooled washed virus corpuscles obtained from four infected rabbits. The word "specimen" applies to elementary bodies obtained from a particular lot and resuspended in a given test solution. An experiment is considered as a single ultracentrifugation of a specimen. Thus numerous specimens were generally prepared from a given stock suspension of elementary bodies, and several experiments were generally done on each specimen. Other terms will be defined as the need arises.

Theory and Equations

When a small particle suspended in a liquid medium is subjected to a constant centrifugal force, the velocity with which it slowly sediments through the liquid is directly proportional to the net force acting on the particle and inversely proportional to the resistance offered by the viscosity of the fluid. If all forces other than the centrifugal force are negligible, the sedimentation velocity V of a particle located at a distance r from the axis of rotation can be expressed, then, as

where ω = speed of centrifuge in radians per second.

v = volume of particle in cc.

σ = average density of particle in gm. per cc.

ρ = density of liquid medium in gm. per cc.

η = viscosity of medium in poises.

K = a constant whose value depends on the amount and shape of the particle's surface which is subjected to the frictional resistance of the liquid.

The sedimentation velocity should be a linear function of ρ if K, v, and σ remain constant. It can become zero in a field of force only when $\sigma = \rho$, regardless of the values of K and v.

For the special case of a spherical particle suspended in a liquid consisting of much smaller particles or molecules, K has the value $K = \dfrac{1}{6\pi a}$, where a is the radius of the particle. This relation is the basis of the well known Stokes' formula as applied to centrifugal fields:

$$V = \frac{dr}{dt} = \frac{2\omega^2 r a^2(\sigma - \rho)}{9\eta} \tag{2}$$

For a slightly oval particle, this equation should be valid to a close approximation if a is taken as the average radius.

The sedimentation velocity in a unit field of force is then

$$\frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{2a^2(\sigma - \rho)}{9\eta} \tag{3}$$

Integrated for a displacement $(r_2 - r_1)$ of the particle during the time interval $(t_2 - t_1)$, the expression becomes

$$\frac{\log_e r_2 - \log_e r_1}{\omega^2(t_2 - t_1)} = \frac{2a^2(\sigma - \rho)}{9\eta} \tag{4}$$

It is customary to describe the behavior of a particle in a field of force by its computed sedimentation constant, S_{w20}, which is the rate at which sedimentation would proceed in a unit field of force if the suspending medium had a density and viscosity equal to those of water at 20°C. However, in order to compare the sedimentation rates of particles of unknown densities in solutions of varying specific gravity, use will be made of a more general term, "corrected sedimentation rate," denoted as S_c and defined by the relation

$$S_c = \frac{\log_e r_2 - \log_e r_1}{t_2 - t_1}\frac{1}{\omega^2}\frac{\eta}{\eta_{w20}} \tag{5}$$

where η_{w20} is the viscosity of water at 20°C.

In accordance with Equation 4, for spherical particles

$$S_c = \frac{2a^2(\sigma - \rho)}{9\eta_{t20}} \tag{6}$$

For dilute buffer solutions, etc., which have densities close to that of water, $S_{w20} = S_c$ to a good approximation.

All sedimentation rates, except where otherwise specified for comparative purposes, will be given in terms of 10^{-11} cm./sec./dyne.

Centrifugation Technique

As soon as possible after a particular sample of elementary bodies had been suspended in the desired medium, a portion of the mixture was placed in the centrifuge cell and the remainder stored in a refrigerator at 5°C. until it was needed for another run. The centrifuge cells used were of two types: One had a hard rubber centerpiece with an aperture 11 mm. in length, and the other had a pontalite[1] centerpiece with an aperture 15 mm. in length. Fresh dry cells were always used except in certain cases where two runs on the same lot of material were made within a short time of each other.

The temperature of the centrifuge rotor was measured by a thermocouple immediately before and after each run. The rise in temperature was seldom more than a few tenths of one degree Centigrade; therefore the mean temperature was used in computing the corrected viscosity of the solution. The speed of the centrifuge was generally adjusted to such a value that seven or eight photographs, spaced at 3 minute intervals, could be taken while the sedimenting boundary traversed about three-quarters of the cell. Speeds ranging from 3600 to 48,000 R.P.M. were used according to the densities and viscosities of the suspending media. The exposure time was usually 15 seconds. All photography was done with ultraviolet light furnished by a mercury arc and filtered through absorption cells of bromine and chlorine gases. The photographs of the sedimentation boundaries were analyzed by means of a continuous recording photomicrometer. The several boundary positions determined for each experiment by this method were used in computing the mean sedimentation rate according to Equation 5.

EXPERIMENTAL

Boundary Measurements.—Sedimentation boundaries produced by the elementary bodies of vaccinia were studied under a variety of experimental conditions. Fig. 1 is a typical photographic record of the sedimentation of Paschen bodies in a dilute buffer solution. The

[1] A new clear, plastic material manufactured by E. I. Du Pont De Nemours and Company.

boundary is seen to become progressively more blurred as it moves through the solution.   The blurring, or spread of the boundary, *i.e.*, the distance between the upper and lower edges of the boundary, is better illustrated by the set of curves shown in Fig. 2. These curves represent the photomicrometer tracings of the photographs constituting Fig. 1.   Each of these tracings indicates the distribution of the particles at a particular stage of the centrifugation.

Experimental as well as theoretical considerations have shown that only a small proportion of the spread of boundary could have been caused by diffusion effects which usually account for the blurring of boundaries in homogeneous solutions of proteins.   The blurring occurred to the same extent when elementary bodies were centrifuged in more viscous solutions of sucrose, glycerol, and urea, instead of buffer solutions. Moreover, the fact that elementary bodies suspended in media of different densities showed no notable variation in the spread of boundaries renders very doubtful any assumption that the particles differ considerably in density.   A small amount of the spread depended on the fact that the boundary moved an appreciable distance during the photographic exposure and also on the fact that the photographic definition was not infinitely sharp.   These factors, however, remained constant during the course of an experiment; therefore, most of the progressively increasing spread must be interpreted as a centrifugal effect.   The spread of boundaries might be explained on the basis of slight variations in the size or shape of the elementary bodies.   However, in view of considerations to be discussed below, it appeared probable that part of the spread was caused by the agglutination or partial aggregation of some of the single particles.

As can be seen both in Figs. 1 and 2, the concentration of virus particles below the boundary is decreasing at what appears to be an approximately uniform rate, except, of course, at the bottom of the cell.   This decrease has been attributed by Svedberg to the fact that as any given group of particles sediments through the wedge-shaped cell, it is progressively subjected to a slightly higher centrifugal force and at the same time is progressively passing into a wider section of the cell.   For an approximately homogeneous group of particles, this decrease in concentration bears a definite relation to the distance

FIG. 1. Sedimentation of elementary bodies of vaccinia in a dilute buffer solution of pH 7.6 containing 2 per cent normal rabbit serum; speed, 4800 R.P.M.; mean centrifugal force, 1670 times gravity; interval between photographs, 3 minutes; exposure time, 15 seconds. *a* and *b* indicate the upper and lower edges respectively of the sedimenting boundary; the distance between constitutes the spread. *M* is the meniscus of the medium and *L* the light intensity standard.
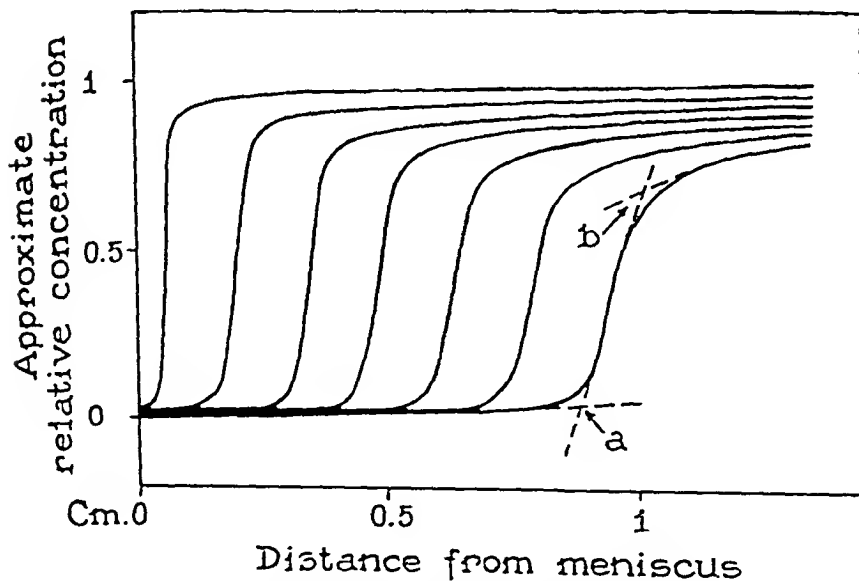
FIG. 2. Sedimentation curves reproduced from the photomicrometer tracings of the photographs shown in Fig. 1. The method of determining the upper and lower edges (*a* and *b*) of the sedimenting boundary is described in the text.

through which the boundary has progressed at any time, and therefore its theoretical value can be computed and used as one indication of the presence or lack of homogeneity.

The upper edge of the boundary, *i.e.*, the slowest moving part, was always more sharply defined than the lower edge; consequently, it has been used for the determination of boundary positions. A method was adopted whereby a tangent was drawn to the distribution curve, obtained by means of the photomicrometer, on either side of the region of highest inflection, as is illustrated in Fig. 2. The intersection of the tangents, represented by point *a* in the figure, indicated the distance, from the meniscus, of the slowest moving vaccine virus particles present in an appreciable concentration. The distance of the lower edge of the boundary from the meniscus was similarly approximated by applying the method of tangents. Point *b* in Fig. 2 indicates the position of the lower edge of the boundary. By repeating the procedure for each of the several curves, the average sedimentation velocities of both the upper and lower edges of the boundary were determined. The difference between these sedimentation rates, expressed in per cent, was employed as a measure of the boundary spread.

The lower edge of a boundary consistently sedimented 12 to 16 per cent more rapidly than the upper edge. This definite amount of spread was characteristic of a sedimenting boundary of elementary bodies, whether the particles were suspended in solutions of dilute buffer alone, or in various concentrations of sucrose, glycerol, or urea.

Sedimentation in Dilute Buffer Solutions.—The results of centrifugation experiments carried out on elementary bodies of vaccinia suspended in dilute buffer solutions having pH values between 6.8 and 7.6, both with and without small amounts of rabbit serum, are summarized in Fig. 3, Chart 1.

Eleven specimens were prepared from seven different stock suspensions of elementary bodies and employed for twenty-five centrifugation experiments extending over a period of 12 months. Several experiments were generally performed on a given specimen. These were done at various intervals after the specimen had been prepared from a particular stock suspension of virus. The suspension time was taken as the interval between the addition of the elementary bodies to the test medium and the first photographic exposure taken during the experiment.

Chart 2

		XIII f 2940		
XVII e 36	XIII f 2880		
XIII f 300	XIII f 63		XVI e 27
XV e 44	XIII f 9	XII e 35	XIV f 320

Chart 1

		X h 80	XI i 15		
		VIII g 280	IX h 75		
		VII g 110	VIII g 410		XI i 1440
		VI g 300	VII g 1200		VIII g 110
	X h 150	V e 250	VI g 90		IV f 240
	IX h 140	V e 200	III f 7000		IV f 66
V e 47	VII g 30	V c 1380	II g 30	I b 30	IV f 8	III f 7060

44 45 46 47 48 49 50 51 52 53 54
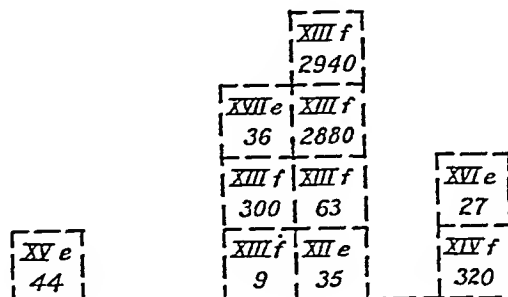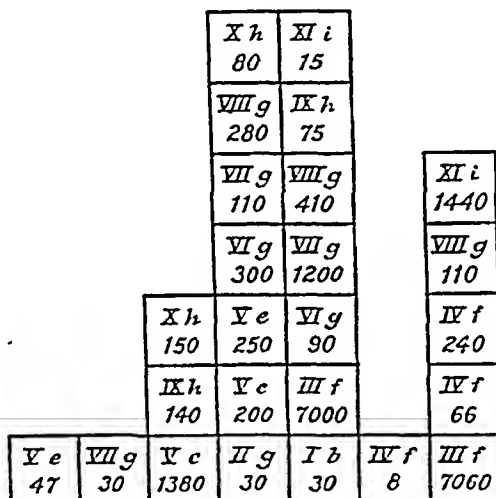
Sedimentation Constant in Units of 10^{-11} cm/sec/dyne

FIG. 3. *Charts 1 and 2.*—Distribution of the sedimentation constants of elementary bodies of vaccinia in media with densities and viscosities near those of water. Each experiment is indicated as a small square. The letter inside the square refers to the lot of stock virus from which the particular specimen was prepared. The Roman numeral refers to the specimen number, while the Arabic numeral indicates the suspension time in minutes. The suspending medium used in Chart 1 was dilute buffer of approximately neutral pH; specimens IV, V, VI, VII, and X contained 2 per cent rabbit serum. The media in Chart 2 had varying salt and serum concentrations and covered a wider pH range.

It is apparent that the distribution of twenty-five sedimentation constants, graphically represented in Fig. 3, Chart 1, approximates a normal probability distribution.   Each value is based on measurements taken of the upper and better defined edge of the boundary. No preferential distribution was shown by these sedimentation constants with respect to different stock suspensions of virus, different specimens, time after suspension, or with respect to the presence or absence of 2 per cent rabbit serum.

The mean sedimentation constant from these twenty-five experiments was computed to be 49.1×10^{-11} cm./sec./dyne, with a probable deviation of 0.5 per cent.   The highest and lowest rates of uniform sedimentation recorded in dilute buffer were 51.7 and 45.3.   Over 90 per cent of the boundaries settled at rates between 47 and 52, only two of them falling below this range.   The correct value for the most probable sedimentation constant of the slower moving elementary bodies of vaccinia in a standard buffer solution must be close to 49.1 $\times 10^{-11}$ cm./sec./dyne, ånd this value is used as the basis of the interpretations to follow.

Sedimentation in Other Media with Densities Near That of Water.— From the experiments just described, dilute buffer solution of about neutral pH and with 2 per cent rabbit serum was found to be a desirable suspending fluid.   Nevertheless, a study of the behavior of elementary bodies in other solutions was indicated.   The distribution of the sedimentation rates measured in ten experiments with solutions of about the density of water but differing from those used in the preceding section is indicated by the series of dotted squares in Fig. 3, Chart 2.

The virus material for the six specimens employed was taken from two of the lots which were used for studies with dilute buffer solutions.   Specimen XII was a suspension of virus in a dilute buffer-serum solution, pH 7.2, to which had been added 0.4 per cent NaCl, while in specimen XIII the salt content was increased to 0.8 per cent.   10 per cent, by volume, of rabbit serum was incorporated in, dilute buffer for the suspending medium of specimen XV.   Specimen XIV was a resuspended sample of specimen IV (Fig. 3, Chart 1) which had been sedimented completely to the bottom of the centrifuge cell and then spun at a speed of 720 R.P.S. for about 15 minutes.   Specimens XVI and XVIII, containing 2 per cent rabbit serum in dilute buffer, had final pH values of 6.2 and 8 respectively.

None of these variations in the conditions of experiment, *i.e.*, subjection to high centrifugal forces or moderate changes in the salt, serum, or pH of the suspending media, produced any change in sedimentation rate, as evidenced by Fig. 3, Chart 2, which might be regarded as significant. However, an interesting observation was made in connection with the specimen which had been suspended in an 0.8 per cent NaCl buffer solution. Within only 9 minutes after preparation the concentration of elementary bodies constituting the primary sedimentation boundary had dropped to a very low value. That the great majority of the virus particles had agglutinated was evidenced by a progressive increase of particle concentration below the sedimenting boundary at every stage of the centrifugation, without visible evidence of discrete multiple boundaries. The marked tendency of elementary bodies to agglutinate in physiological salt solution is well known (16); however, macroscopic agglutination is not generally apparent in such a short time. The concentration of single particles continually decreased in several experiments with this specimen over a period of 48 hours until barely a trace of a primary boundary could be detected. However, another experiment performed on the same specimen only 1 hour later (*i.e.*, suspension time, 49 hours) showed a higher concentration of single particles. This, perhaps, indicated a spontaneous partial reversal of agglutination. Such partial reversal of agglutination of elementary bodies has not been considered previously, mainly because the existing methods were inadequate for its detection unless the effect were very marked.

Multiple Boundaries.—In addition to the primary sedimentation boundary, more rapidly moving boundaries were recorded in several instances. In no case did the presence of such boundaries appear to influence the sedimentation rate of the principal boundary. An example of multiple boundaries is illustrated by the photographs in Fig. 4 and the corresponding sedimentation curves in Fig. 5. A second boundary is clearly defined, and there are distinct traces of even a third. In Fig. 5 the three boundaries are indicated, in order, as 1, 2, 3. As the figure shows, the transition in concentration of material from one boundary to the next is rather gradual, and the main boundary continues to be better defined than the others. The method of measurement which has already been outlined was success-

FIG. 4. Multiple boundaries in a sedimenting suspension of elementary bodies of vaccinia.
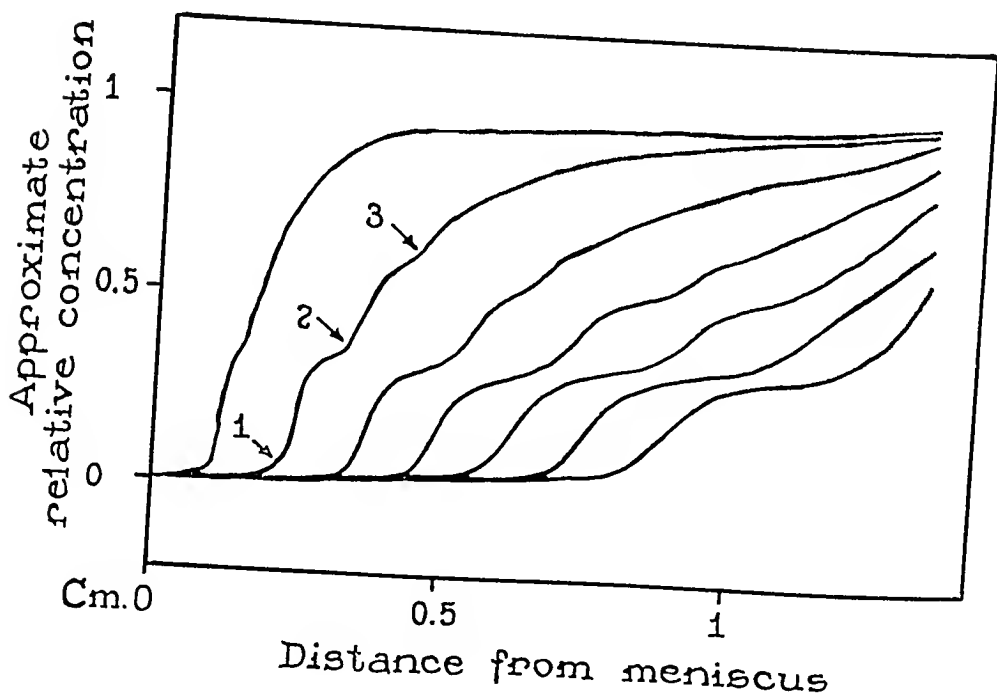
FIG. 5. Sedimentation curves reproduced from the photomicrometer tracings of the photographs shown in Fig. 4. The upper edges of the primary, secondary, and tertiary boundaries are indicated on the second curve.

fully applied in a number of cases to approximate the sedimentation rates of these more rapidly moving particle groups.

Secondary boundaries were evident in at least twenty-five of the 153 experiments performed in connection with the present studies. Definite tertiary boundaries were apparent in nine of these, and in one instance a fourth boundary could be detected. The concentration of particles constituting a primary boundary was in all instances higher than the concentration of those constituting the accompanying secondary boundary. The secondary in turn represented a higher concentration than the tertiary boundary. Thirteen secondary boundaries were

TABLE I

Characteristics of Secondary Boundaries

Virus lot	Specimen	Suspension time	Suspending medium	Approximate per cent of total virus in 1st and 2nd boundaries		Ratio of sedimentation rates S_2/S_1
				S_1	S_2	
		min.		*per cent*	*per cent*	
ε	VII	30	Dilute buffer	70	15	1.52
ε	VII	110	" "	45	25	1.47
ε	VII	1200	" "	30	20	1.43
ε	XII	35	0.4 per cent NaCl + buffer	60	20	1.46
ε	XVI	27	Dilute buffer	50	20	1.47
ε	XVIII	190	13 per cent sucrose	50	30	1.57
ε	XVIII	260	" " " "	60	25	1.52
ε	XVIII	1380	" " " "	30	25	1.56
ι	XIX	7	Dilute buffer*	20	15	1.51
ι	XX	180	" " *	45	15	1.50
ι	XX	1380	" " *	35	15	1.57
ƒ	XXI	10	" " *	60	15	1.37
ƒ	XXI	55	" " *	60	20	1.45

* Virus sedimented from urea solution and resuspended in dilute buffer solution.

sufficiently well defined to permit approximate measurements of their sedimentation rates. These are compared with the corresponding principal boundary rates in Table I. On the average the secondaries sedimented about 1.49 times as fast as the primary boundaries. The highest and lowest ratios recorded were 1.57 and 1.37 respectively. 70 per cent of the ratios lay between 1.43 and 1.51. A few measurements indicated a sedimentation rate for the tertiary boundaries of approximately 1.85 times that of the primary.

Little definite correlation has been established between the conditions of experiment and the appearance of multiple boundaries.

Only half of the lots of virus displayed the phenomenon, although it is probable that any lot was potentially capable of doing so.

The first specimen prepared from lot *b* showed a secondary boundary when centrifuged in a dilute buffer solution. A number of subsequent preparations obtained from the same lot and suspended in sucrose solutions all showed irregular sedimentations of a nature to be discussed below. Lot *e* displayed only primary boundaries during a number of experiments extending over a period of several weeks. However, when the pH value of a dilute buffer suspending medium was lowered to 6.2, a double boundary appeared. Another experiment performed a few hours later on the same lot suspended in a similar solution having a pH of 8 showed no evidence of the phenomenon. Several more experiments were performed on this same lot using glycerol solutions as suspending media; only primary boundaries were in evidence. However, a few days later another suspension of the same lot *e* in a 0.4 per cent NaCl buffer solution displayed a secondary boundary. It is worthy of note, in this connection, that a double boundary was produced by suspending the virus particles in a 0.4 per cent NaCl solution, whereas raising the salt content to 0.8 per cent in the suspending medium of specimen XIII resulted in an agglutination of most of the particles without the evidence of multiple boundaries. Lot *g* likewise showed only primary boundaries over a period of several weeks when suspended in dilute buffer and in various concentrations of glycerol. Then it began to show multiple boundaries consistently when suspended in an identical buffer solution and also when suspended in various concentrations of glycerol and sucrose.

The only other multiple boundaries recorded could be classed as falling under a special set of conditions. In five separate instances elementary bodies were centrifuged from various solutions of sucrose, glycerol, and urea and resuspended in a dilute buffer-rabbit serum solution. In every instance resuspension was followed by the appearance of secondary boundaries. The effect was most pronounced with resuspensions from urea solutions and least pronounced with resuspensions from sucrose solutions. Specimens XIX, XX, and XXI, which are listed in Table I, fall into this classification.

In only one instance did the presence or absence of secondary boundaries fail to be consistent throughout a series of experiments performed on a particular specimen. A sample of a specimen resuspended from a 33 per cent glycerol solution showed both primary and secondary boundaries after 6 minutes, but only the primary boundary was present in the sample run after 1 hour. Both boundaries were noted again after 1 hour and 50 minutes. The concentration of the single particles increased from about 55 per cent of the total concentration in the first experiment to 75 per cent in the last experiment. In general, however, a given specimen showed a decrease in the concentration of the principal boundary on long standing (see Table I, specimens VII, XVIII, and XX). This was associated with microscopic, and in some instances macroscopic, evidence of

agglutination of elementary bodies.   Nevertheless, instances such as the one just mentioned were recorded of spontaneous small increases in the concentration of the single particles several hours after suspension.   This probably represents dispersion of small aggregates of elementary bodies.

Multiple boundaries were not always equally well defined.   Thus, in a few instances they were more sharply differentiated than those in Fig. 4, while in many cases they were barely distinguishable.   In these latter cases it was debatable whether to attribute the character of the photomicrometer tracings to the presence of multiple boundaries or to the presence of aggregates composed of various numbers of elementary bodies, all of which were sedimenting more rapidly than the particles constituting the primary boundary.   Specimens that had multiple boundaries generally gave evidence, in the photomicrometer curves, of a considerable amount of aggregated material which settled more rapidly than any of those particles constituting a boundary.   Microscopic examination of stained smears and dark-field examination of specimens showing multiple boundaries have confirmed the presence of aggregated groups containing various numbers of elementary bodies.

Irregular Boundary Movements.—The groups of sedimentation rates presented in Fig. 3, Charts 1 and 2, were based, with only one exception, on boundaries moving at uniform rates through the suspending media.   A photographic record of sedimentation obtained with specimen II indicated a non-uniform sedimentation velocity; the boundary alternately speeded up and slowed down throughout the centrifugation.   The average sedimentation rate for this specimen was found to be far above that of any other specimen included in Fig. 3, Chart 1, although the shape of the photomicrometer curve at any stage of the centrifugation was not greatly different from the others.   On the other hand, when the sedimentation velocity was determined from only those two consecutive curves which would give a minimum value, an approximately normal value was obtained. It appeared, therefore, that the boundary was behaving in an approximately normal manner only when it was moving slowest.   To reduce experimental error in the approximation of such minimum rates, the sedimentation velocity was taken as the mean of the determinations on two pairs of curves.   The sedimentation rate given in Fig. 3,

Chart 1 for the one experiment with specimen II is a minimum rate determined as just described.

Irregular boundaries similar to the one just described occurred in thirty-three experiments in which various concentrations of sucrose were employed. Their appearance was confined to four of the ten stock suspensions of elementary bodies used. These four lots of virus were investigated only in dilute buffer and in sucrose solutions. They were quite consistent in their abnormal behavior, although in some instances the boundary appeared almost normal. In every case
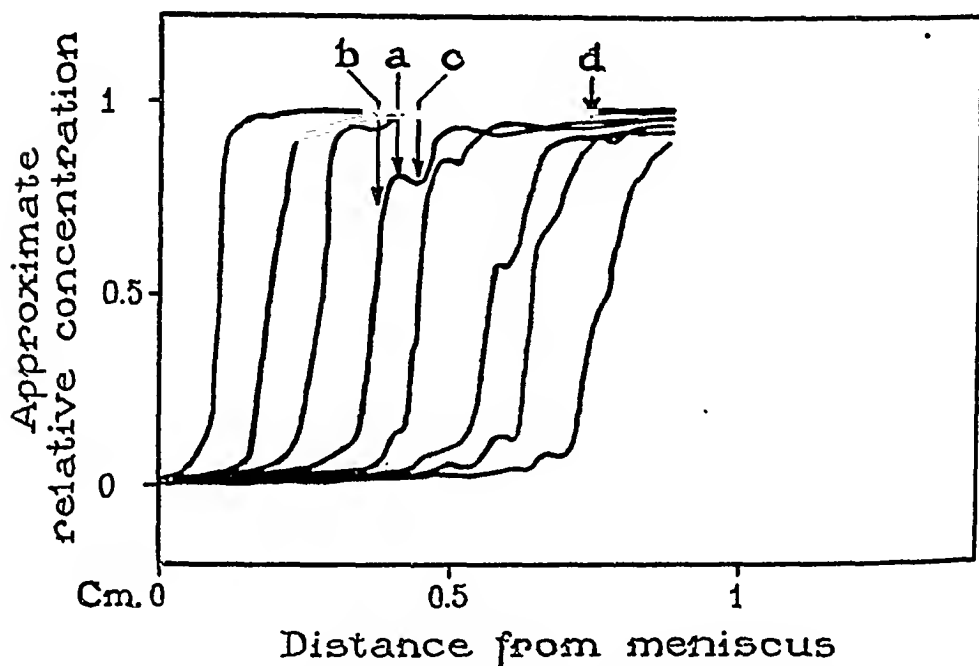
Fig. 6. Irregular sedimentation of elementary bodies. See text, page 597, for interpretation of sedimentation curves.

the minimum rate approximated the normal rate as determined with more stable suspensions. As an example of abnormal behavior, the average rate of sedimentation for a particular boundary was computed to be about 1.6 times its minimum rate, which was approximately normal. Its maximum rate, as determined from three of the photomicrometer curves, was 2.2 times the normal rate.

Many of these irregular boundaries were readily identified by the characteristic striated appearance of their photographs. A typical example is illustrated by the series of photomicrometer tracings in Fig. 6. The irregular spacing and broken appearance of the distribu-

tion curves are at once evident and distinguish them from the curves shown in Figs. 2 and 5.

A peculiar feature was observed on several occasions in specimens with irregular boundaries. The concentration of particles at some particular level (a, Fig. 6) in the cell was actually greater than the concentration of material at levels just above b and below c. This condition does not persist, probably, in part at least, because the slightly heavier layer tends to displace the less concentrated layers below it. This phenomenon never occurs in the course of a normal sedimentation, even of complex mixtures, and it can be explained plausibly only by assuming at least one of three possibilities: (1) Some of the particles which had been at the level c had experienced a spontaneous increase or decrease in their sedimentation rate; (2) Some of the material corresponding to level a had suffered a decrease in sedimentation rate; (3) Or some of the particles corresponding to level b had increased their settling rate. The last of these possibilities seems the most probable. A spontaneous increase in the sedimentation velocity of particles near the boundary could result from agglutination of elementary bodies which would account for these negative concentration gradients.

Still another distinguishing feature connected with irregular boundaries was the piling up of particles in the regions below the boundary; thus the regular and progressive decrease in concentration that occurred during the centrifugation of homogeneous material was absent. For instance, in Fig. 6 the concentration curves are close together near the level d, and some of them cross each other, indicating an actual increase in the concentration of material in some regions at various stages of the centrifugation. (Compare with Figs. 2 and 5.) This abnormality can be explained best by postulating a more rapid sedimentation, on the average, for those particle groups in the region of the boundary than was experienced by the mass of particles constituting the suspension.

It was found, as has already been mentioned, that irregular boundaries of this type could generally be avoided by a more careful selection of the lots of stock virus.

In only one recorded instance was the primary boundary replaced or accompanied by an appreciable amount of ultraviolet light-absorbing material which sedimented slower than the normal rate:—

During the course of 1 day, three experiments were performed on a specimen of lot g which had been suspended in a 59 per cent glycerol solution. The primary boundaries appeared normal, but were accompanied by more rapidly moving

boundaries and a considerable quantity of large aggregates which rapidly sedimented out of the field. The sedimentation rate, corrected for viscosity, was consistently about 28 (17). However, when the same specimen was again centrifuged the following day, the peculiar sedimentation record illustrated in Fig. 7 was obtained. There was no well defined boundary present during the early stages of the centrifugation, but one did appear later which could be measured approximately. Its rate was estimated to be about 20 during the sedimentation between levels a and b, as compared to 28, the normal in this medium. A bound-
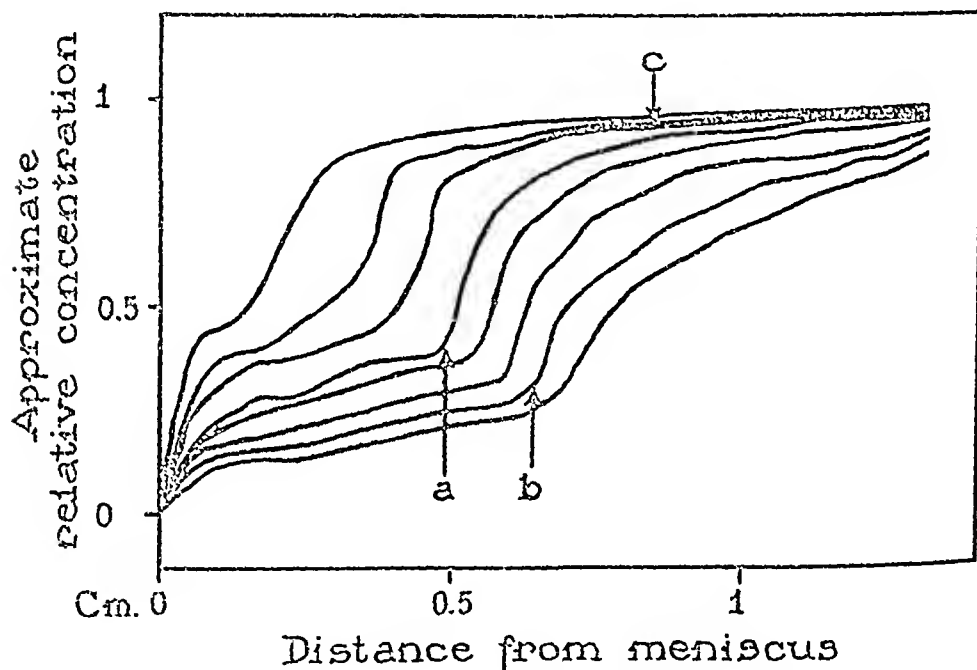
FIG. 7. Sedimentation curves for the single specimen of elementary bodies whose sedimentation rate was appreciably lower than normal. See text, page 599, for discussion.

ary moving at this rate could not have progressed from the meniscus to the indicated positions during the respective time intervals of centrifugation. It must, therefore, have originated at some stage during the centrifugation and consequently could not represent a group of particles which had remained unchanged since the beginning of the experiment. It is to be noted that in Fig. 7 the first three curves lie close together at point c, as did the curves at point d in Fig. 6. This again indicates abnormal concentrations in the lower portions of the medium. A similar set of experiments with a specimen taken from virus lot c did not show any of the abnormalities observed with the specimen just discussed.

Estimation of Particle Size.—The most probable sedimentation constant for the slower moving single virus particles present in an appreciable concentration has been computed to be 49.1×10^{-11} cm./sec./dyne. From the results reviewed elsewhere (17) the particle density has been determined as 1.16 gm. per cc. From these values, the mean diameter of the elementary bodies of the CL dermal strain of vaccine virus was estimated at 236 mμ, on the reasonable assumption of an approximately spherical shape. Allowing for the possibility that the observed 14 per cent spread in the sedimentation boundary might be due to a variation in the size of the elementary bodies, the average particle diameter was computed to lie within the range 236 mμ to 252 mμ.

DISCUSSION

The Nature of Primary Boundaries.—Studies on the elementary bodies of the CL dermal strain of vaccine virus by means of the analytical ultracentrifuge indicate that under the standard conditions of experiment, virus particles obtained from a properly prepared and selected material generally sediment with the formation of a single boundary. Such a boundary is not sharply demarcated but always exhibits a definite amount of blurring or spread. The major portion of this spread apparently is accounted for by slight differences in the size or shape of the particles or by a slight degree of agglutination among some of the particles; perhaps all three factors play a part. The spread of the boundaries generally lies between 12 and 16 per cent. Sedimentation rates calculated from the movement of these primary boundaries were consistent for different lots of virus suspended in various solutions having approximately the same density and viscosity as water when the pH values of the solutions lay between 6.2 and 8. In these media the mean sedimentation constant of the slowest moving virus particles present in an appreciable concentration was found to be 49.1×10^{-11} cm./sec./dyne, with a probable deviation of 0.5 per cent.

Characteristics of Multiple Boundaries.—Under conditions not yet strictly defined, multiple boundaries of elementary bodies of vaccinia may appear on centrifugation. Lowered pH values (6.2), medium concentrations of salt (0.4 per cent NaCl), and relatively long storage of stock suspensions appear to favor the phenomenon. Some lots of

material are more apt than others to show the effect, although it may not invariably recur when several centrifugations are made on the same lot. Multiple boundaries were consistently produced when Paschen bodies which had been stored in a concentrated solution of urea for a number of hours were resuspended in a dilute buffer solution.

Virus particles constituting the primary boundary were always present in a higher concentration than those constituting an accompanying secondary boundary; an analogous rule also governed the content of the secondary boundaries with respect to that of the tertiaries. This fact suggests that the production of each of the more rapidly moving particle groups depends on, and is in some way proportional to, the concentration of particles constituting the slower moving boundaries.

According to Stokes' law, if the masses of two identical spherical particles were combined to form a larger spherical particle having the same density, its sedimentation velocity in a gravitational field of force would be 1.58 times the velocity of the two original particles. Three particles so combined would increase their rate by a factor 2.07. In either case the ratio could be anything between unity and the value cited if the larger particle formed had some shape other than spherical. For example, two non-rigid particles could conceivably be drawn into a close enough combination by the action of surface forces to increase their potential sedimentation rate by a factor closely approaching 1.58. It is interesting to note in this connection that secondary boundaries of Paschen bodies generally sediment about 1.50 times as rapidly as their primary boundaries; moreover, the tertiary boundaries usually move about 1.85 times more rapidly than the primary. The possibility that multiple boundaries are dependent on the close combination of 2, 3, or 4 elementary bodies is consistent with many microscopic examinations made on stained and dark-field preparations of these suspensions of vaccine virus.

Interpretation of Irregular Boundaries.—A few stock suspensions of elementary bodies consistently displayed a non-uniform sedimentation rate during the course of an individual experiment. In every instance in which the average sedimentation rate was found to be abnormally high, it was observed also that variations in the actual

rate occurred during the course of the experiment.. This could not be accounted for by experimental error alone. In such an instance the lowest momentary rate recorded over a short period of the total centrifugation time was more nearly characteristic of a normal rate. To be fully aware of such misleading behavior on the part of the sedimenting particles, or to detect the presence of multiple boundaries, it is necessary to record the distribution of the particles at frequent intervals during the centrifugation. For meeting such requirements no simpler technique appears to offer the advantages of the Svedberg photographic method.

A study of the photomicrometer curves representing the irregular boundaries recorded has led to the conclusion that their intermittent acceleration is most probably caused by an autoagglutination of elementary bodies in the region of the boundary itself. The reason for the exaggerated tendency of certain virus suspensions to agglutinate, even with storage alone, is not known. However, if this observation is accepted, then it may be postulated that in the region of the primary boundary centrifugation disturbs some existing statistical equilibrium between single and aggregated particles, permitting accelerated agglutination. Stock virus suspensions which did not give macroscopic evidence of autoagglutination after being stored for several weeks did not demonstrate the phenomenon of irregular sedimentation.

In only one of 153 experiments was there evidence of an appreciable quantity of material that sedimented at significantly slower rates than the normal for the suspending medium employed. Although a possible splitting of the elementary bodies into smaller fragments, or a partial dissolution, must be considered here, an equally acceptable explanation for the retarded sedimentation of the observed boundary might be based on a change in the size or shape of the particles.

Comparison with Previous Investigations.—Previous efforts to assign a definite numerical value to the diameter of vaccine virus have resulted in considerable variation. The methods used for these determinations have fallen into three general groups; namely, centrifugation, filtration, and direct measurement by photomicrography employing ultraviolet light. Earlier experiments have been adequately commented upon by other authors; in this presentation only

a correlation between the more recent results and the present observations will be undertaken.

The use of the analytical centrifuge eliminates two important sources of error which may have entered into previous centrifugation experiments. These are firstly, the difficulties in determining small differences in the amount of virus present when the agent is demonstrated by the infective titer of the material, and secondly, the effect of convection currents in the medium during centrifugation. Further possible sources of error with simpler methods of analysis are apparent from the occasional peculiar behavior of the elementary bodies used in the present studies.

Whatever the relative rôle played by these factors, various workers have used techniques of a certain general type and have obtained centrifugation data that were in many respects reproducible. Thus Bechhold and Schlesinger (10) considered the density of vaccine virus to be 1.10 gm. per cc. and on this basis estimated the diameter of the particle as 210 mμ to 230 mμ. Elford and Andrewes (11) determined the density of vaccine bodies as 1.18 gm. per cc., and by applying this figure to their centrifugation data, they estimated the size of the particle as 170 mμ to 180 mμ. However, they cautioned that this must be regarded as a minimum value. Moreover, these latter workers recalculated the results of Bechhold and Schlesinger, using the value 1.18 gm. per cc. for the density and found that with this figure the size would be 160 mμ to 180 mμ. The sedimentation constant of vaccine virus in broth calculated from the data of these authors (11) would be of the order of 31×10^{-11} which is significantly lower than results with the air-driven centrifuge would indicate. McIntosh and Selbie (12) employed a slightly different centrifugation technique from their predecessors (11); they assumed the density of the vaccine particle to be 1.25 gm. per cc. and estimated the diameter to be 99 mμ to 240 mμ. During the course of the present work a brief report (18) appeared, assigning a sedimentation constant of 54×10^{-11} cm./sec./dyne to elementary bodies of vaccine suspended in dilute buffer solution.

The determination of particle size by means of filtration through graded collodion membranes (19) has proved a valuable laboratory procedure. The diameter of vaccine virus has been determined by

this technique. Elford and Andrewes (20) estimated the size as 125 mμ to 175 mμ, and Païc and coworkers (21) placed the diameter at 140 mμ to 160 mμ. Earlier filtration experiments had indicated a slightly higher value, around 200 mμ (10). Experiments of this type are conducted in the following manner: Virus suspensions are passed through membranes of varying pore size and the end point is taken as the largest pore size that completely withholds the virus. The simple analogy of sand passing through a wire mesh is not applicable here for it is a well recognized fact that the membrane pores must be considerably larger than virus particles to permit their passage. Because of this, a correction factor has been applied to the end point for estimating the size of the particles. The present work suggests that the correction factor in current use may tend to underestimate the size of vaccine bodies.

Barnard has used his photomicrographs taken with ultraviolet light to calculate the size of particles of a neurotesticular strain of vaccine virus. He estimates the diameter of the virus as 160 mμ to 170 mμ according to the publication of Elford and Andrewes (11) and also in a personal communication. There is no obvious explanation for this difference between his results and ours.

CONCLUSION

Ultracentrifugal studies on the CL dermal strain of vaccine virus indicate the following characteristics of the elementary bodies:—

1. A stable suspension of Paschen bodies in a dilute buffer solution of pH 6.2 to 8 sediments with the formation of a characteristic primary boundary which consistently shows a spread of approximately 14 per cent.

2. The principal sedimentation boundary is accompanied frequently by one or several more rapidly moving boundaries which probably are produced by groups of agglutinated elementary bodies consisting of two or more particles.

3. Occasionally the principal boundary may exhibit an irregular or peculiar behavior, a fact which necessitates a careful selection of material and the performance of many experiments for accurate interpretation of results.

4. The sedimentation constant of the slowest moving particles

forming the principal boundary is computed to be 49.1×10^{-11} cm./sec./dyne. On the basis of this sedimentation rate, the average diameter of the smallest virus particles in appreciable amounts is estimated at 236 mμ. If the boundary spread is due principally to slight differences in particle size, then the largest single elementary bodies are approximately 252 mμ in diameter.

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ULTRACENTRIFUGATION STUDIES ON THE ELEMENTARY BODIES OF VACCINE VIRUS

II. THE INFLUENCE OF SUCROSE, GLYCEROL, AND UREA SOLUTIONS ON THE PHYSICAL NATURE OF VACCINE VIRUS

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It has been shown by chemical analysis (1) that elementary bodies of vaccinia in the dry state have approximately the same composition as desiccated bacteria or protoplasm. On the basis of nitrogen determination, 83 per cent of the dry weight of the virus particles appears to be protein. The amount of water present in the elementary bodies in their native state has not been estimated, however. Although the values reported by different investigators (2–4) for the density of vaccine virus are not in agreement, they are all considerably lower than the density of most proteins, namely, 1.33 gm. per cc.

The most suitable method for determining the density and the probable size of virus particles is ultracentrifugation by means of which measurement of sedimentation rates of the particles is made in suspending media of different specific gravities. The elementary bodies of vaccinia possess several advantages for studies by centrifugation: they are approximately spherical in form (5), a fact which greatly simplifies the interpretation of the measurements; they can be purified and concentrated with ease; they are large enough to be sedimented rapidly even in highly viscous media and can be readily observed by dark-field illumination or by proper staining techniques.

In connection with centrifugation studies on particles suspended in different media, it is important to know to what extent the physical nature of the particles (density, size) has been altered by the different

media. Therefore, it is of considerable interest to know whether Paschen bodies behave as semipermeable masses which respond to osmotic influence and whether they exhibit permeability responses to substances other than water. On physicochemical grounds one may expect semipermeable masses to be less permeable to large molecules and more permeable to small molecules if the molecules are non-electrolytes. Accordingly, sucrose, glycerol, and urea, which can be used to vary the specific gravity and osmotic pressure of aqueous solutions, were chosen for the investigation, by means of the ultracentrifuge, of changes in the density and size of vaccine virus particles. The results of the work are reported at this time.

Materials and Methods

Various concentrations of sucrose, glycerol, and urea (all of c.p. grade) were employed as suspending media for investigating the permeability and osmotic response of elementary bodies of vaccinia by means of the ultracentrifuge. The CL strain of dermal vaccine virus used throughout the experiments has been carried in rabbits by dermal inoculation of elementary bodies for the past 4 years in Dr. Rivers' laboratory at The Rockefeller Institute. Sedimenting boundaries of virus particles were obtained by means of the air-driven centrifuge of Bauer and Pickels (6), and the light-absorption method of Svedberg (7) was employed for the measurement of sedimentation rates. The methods employed in the present work for the preparation of virus suspensions and suspending media, as well as the technique of ultracentrifugation, have been described elsewhere (8).

Ten different stock suspensions of elementary bodies were used; seven of these lots furnished the material for thirty-seven parallel experiments with dilute buffer (8). All suspending media used in the present work were so buffered as to have pH values between 6.8 and 7.6, and all centrifugations were made at room temperature. The stock suspensions of virus and the experimental specimens were kept in storage at 5°C. except during the time required for preparation and sampling.

The general nature of the sedimenting boundaries of elementary bodies in solutions of sucrose, glycerol, and urea was found to be in accord with that observed in experiments with dilute buffer (8), and the same methods of measurement were found applicable. The sedimentation rates were computed from measurements taken of the slowest moving virus particles present in an appreciable concentration, i.e., of the upper and better defined edge of the primary boundary. The primary boundary always showed a spread of about 14 per cent (8) which could not be accounted for by diffusion. Each sedimentation rate was corrected to a value representing sedimentation in a medium having a standard viscosity, i.e., that of water at 20°C. Such a corrected sedimentation

rate affords a more favorable basis for the comparison of results obtained with media of different viscosities.

Preparations of elementary bodies were examined microscopically by means of dark-field illumination and also after having been stained according to Morosow's technique (9). The infective titers of certain specimens were obtained by intradermal injection into rabbits of serial tenfold dilutions; duplicate or quadruplicate titrations were done on each sample.

<div align="center">EXPERIMENTAL</div>

Sedimentation of Elementary Bodies in Different Concentrations of Sucrose, Glycerol, and Urea.—The rate at which homogeneous, small particles sediment in a given medium is directly proportional to the difference between the density of the particles and the density of the surrounding medium. If the particles remain constant in size, shape, and density, their sedimentation rates, corrected for the viscosity of the medium, should decrease in direct proportion to the increase of the specific gravity of the suspending liquids. Thus a graph of the corrected sedimentation rates plotted against the specific gravities of different media should give a straight line if the physical nature of the sedimenting particles has not been altered by the suspending fluids. This simple method of analysis was chosen to determine whether or not elementary bodies of vaccinia are appreciably affected by suspension in solutions of sucrose, glycerol, and urea.

The sedimentation rate of elementary bodies was determined at various intervals of time after suspension in several concentrations of these media. Three or four experiments were generally performed on a single specimen of material. The first measurements were taken as soon as possible after the suspension of the virus particles in the test medium, *i.e.*, within 25 to 30 minutes in the experiments with sucrose and glycerol. Later, in the experiments with urea this time was reduced to about 6 minutes by the elimination of a preliminary centrifugation which was found to be unnecessary (8). With the sucrose and glycerol solutions, centrifugations were performed as late as 24 hours after suspension of virus particles in the given media. The behavior of elementary bodies in certain of the urea solutions was investigated as late as 119 hours after suspension. In most instances at least two different specimens of elementary bodies taken from different stock suspensions of virus were employed with a particular concentration of each medium.

Sedimentation rates were measured in sucrose solutions adjusted to specific gravity values of 1.050, 1.100, 1.150, 1.200, and 1.250. All the specific gravity measurements given in this paper are referred to water at 20°C. With these

respective media, thirteen, fifteen, seven, six, and five centrifugations were performed. With glycerol solutions of specific gravities 1.050, 1.081, 1.100, and 1.150, eight, four, eight, and seven experiments, respectively, were performed. Elementary bodies suspended in urea solutions of specific gravities 1.025, 1.043, 1.060, and 1.100 were studied by means of six, three, fifteen, and three centrifugations, respectively.

In the forty-six experiments with sucrose solutions, six different stock suspensions of elementary bodies were employed. Four of these lots of stock virus, used exclusively for the first thirty-five centrifugations, displayed varying degrees of irregularity in their sedimentation because of spontaneous, intermittent agglutination of some elementary bodies during the course of experiment. The sedimentation rates of these specimens were determined by the method outlined in another paper (8) and were found to show a fair agreement under similar experimental conditions. To confirm these results, eleven representative experiments were repeated with two carefully selected suspensions of stock virus which had been found to exhibit only normal boundaries in dilute buffer. In all subsequent investigations only those lots of virus were used which had shown no macroscopic evidence of autoagglutination after storage for about 3 weeks (8). Furthermore, 2 per cent normal rabbit serum was included in most of the suspending media to reduce the possibility of agglutination.

Of special significance were the results obtained with sucrose solutions of specific gravity 1.25. This was the only medium in which there was no evidence of sedimenting boundaries of elementary bodies, even though the centrifugal force was raised to a value (speed, 800 R.P.S.) 100 times greater than that ordinarily used (speed, 80 R.P.S.) with dilute buffer solutions. Five experiments were performed with three different lots of virus and each gave the same general result, although the time of suspension ranged from 30 minutes to 24 hours. In a few experiments there were definite traces of a very slow negative sedimentation without the formation of a distinct boundary. In the sample suspended for 24 hours there were faint traces of both positive and negative sedimentations being experienced simultaneously by small amounts of ultraviolet light-absorbing material. The results were quite consistent, however, in that a sedimentation rate of approximately zero was usually obtained. This means that the density of elementary bodies of vaccinia is very close to 1.25 gm. per cc. when the particles are suspended in sucrose solutions of that density.

The sedimentation rate of a sample of elementary bodies did not remain constant after suspension in a solution of sucrose, glycerol, or urea, but showed a variation which will be discussed below. The maximum corrected sedimentation rate of the bodies for each medium is represented graphically in Fig. 1. Each of these values is based on the results obtained with at least two stock suspensions of elementary bodies, except in the cases of urea solutions of specific gravities

1.043 and 1.100 and of the glycerol solution of specific gravity 1.081. It is immediately evident from this graph that the sedimentation rates do not fall on a straight line and that the behavior of elementary
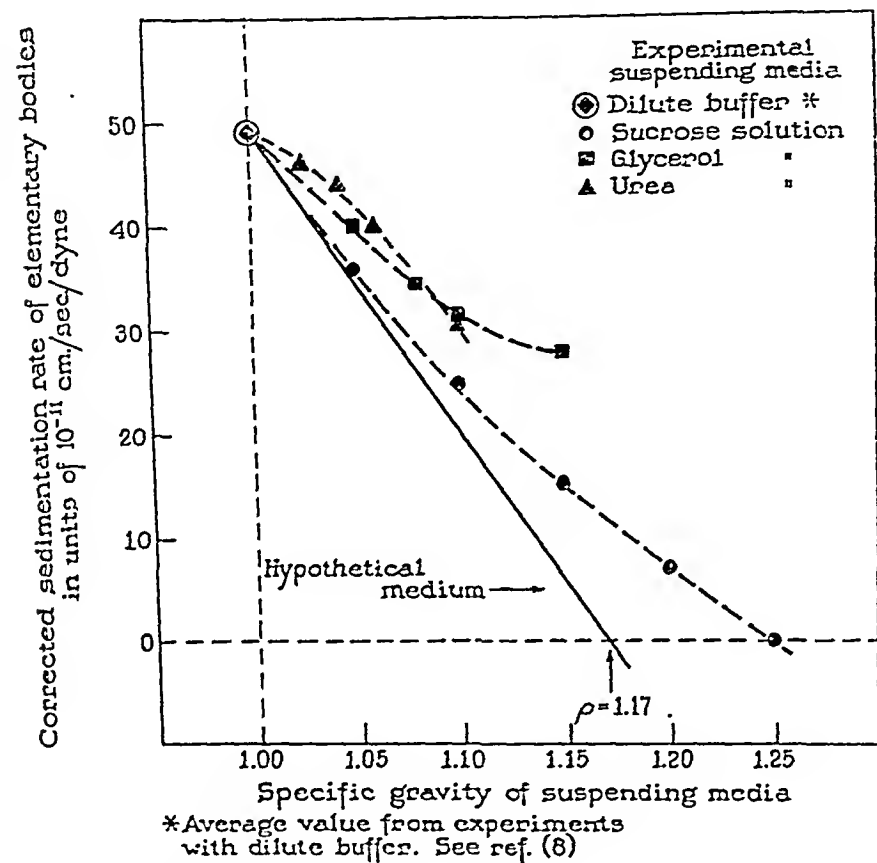
Fig. 1. Maximum sedimentation rates (mean values, corrected to standard viscosity) occurring after suspension of elementary bodies of vaccinia in various concentrations of sucrose, glycerol, and urea. A maximum possible density of elementary bodies suspended in dilute buffer is indicated by the specific gravity (1.17) of the hypothetical medium (tangent line) at which the sedimentation rate becomes zero. For explanation of hypothetical medium see text, page 620.

bodies was quite different in the three types of media employed. From this observation it may be concluded that solutions of sucrose, glycerol, and urea produce changes in the physical nature of the elementary

bodies which result in variations of their sedimentation rates.   It is also obvious that the physical changes produced in the particles by these media are not the same even when the specific gravities of the different solutions are equal.

Rise and Fall of Sedimentation Rate Related to Time after Suspension of Elementary Bodies in Test Solutions.—It has been previously reported that no significant change occurred in the sedimentation rate of Paschen bodies following their resuspension in dilute buffer solutions (8).   In contrast to this, a definite change in the sedimentation rate generally was observed after the suspension of elementary bodies in a given solution of either sucrose, glycerol, or urea.   This variation was found to be correlated with the length of time that the elementary bodies were in contact with the test solution.   The sedimentation rate increased in almost every instance immediately after suspension of the virus particles, and in certain cases a subsequent decline in rate was observed after several hours.   The nature of these variations in sedimentation rate is demonstrated in Figs. 2, 3, and 4.

The results of experiments performed with sucrose solutions are represented graphically in Fig. 2.   Many of these rates were determined with stock suspensions of elementary bodies which displayed in varying degree an irregular sedimentation.   However, it was possible, as has been described previously, to approximate the correct sedimentation rates in such instances, and these values served to confirm the determinations made with stock suspensions which showed only uniform sedimentation.   Where possible, these latter rates were used in drawing the curves (Fig. 2) to indicate the most probable behavior of the elementary bodies at various times after suspension in the several concentrations of sucrose.

The sedimentation rates obtained with glycerol solutions are represented in Fig. 3.   Two specimens of virus particles, taken from two different stock suspensions of elementary bodies, were employed in studies connected with each concentration of glycerol, except the solution of specific gravity 1.081.   Although the maximum rates reached by the two lots of virus agreed closely in every instance, some difference in behavior was observed in the centrifugations made immediately after suspension and those made after about 24 hours.   As may be seen in Fig. 3, virus lot *c* consistently gave lower sedimentation rates immediately after suspension than did lot *g*.   In the solutions of specific gravity 1.15 the elementary bodies taken from virus lot *c* showed a steadily increasing sedimentation rate over a period of 24 hours, reaching a maximum of about 28×10^{-11} cm./sec./dyne.   In contrast to this, the corpuscles from lot *g* sedimented at an approximately uniform rate of 28 during the course of three experiments
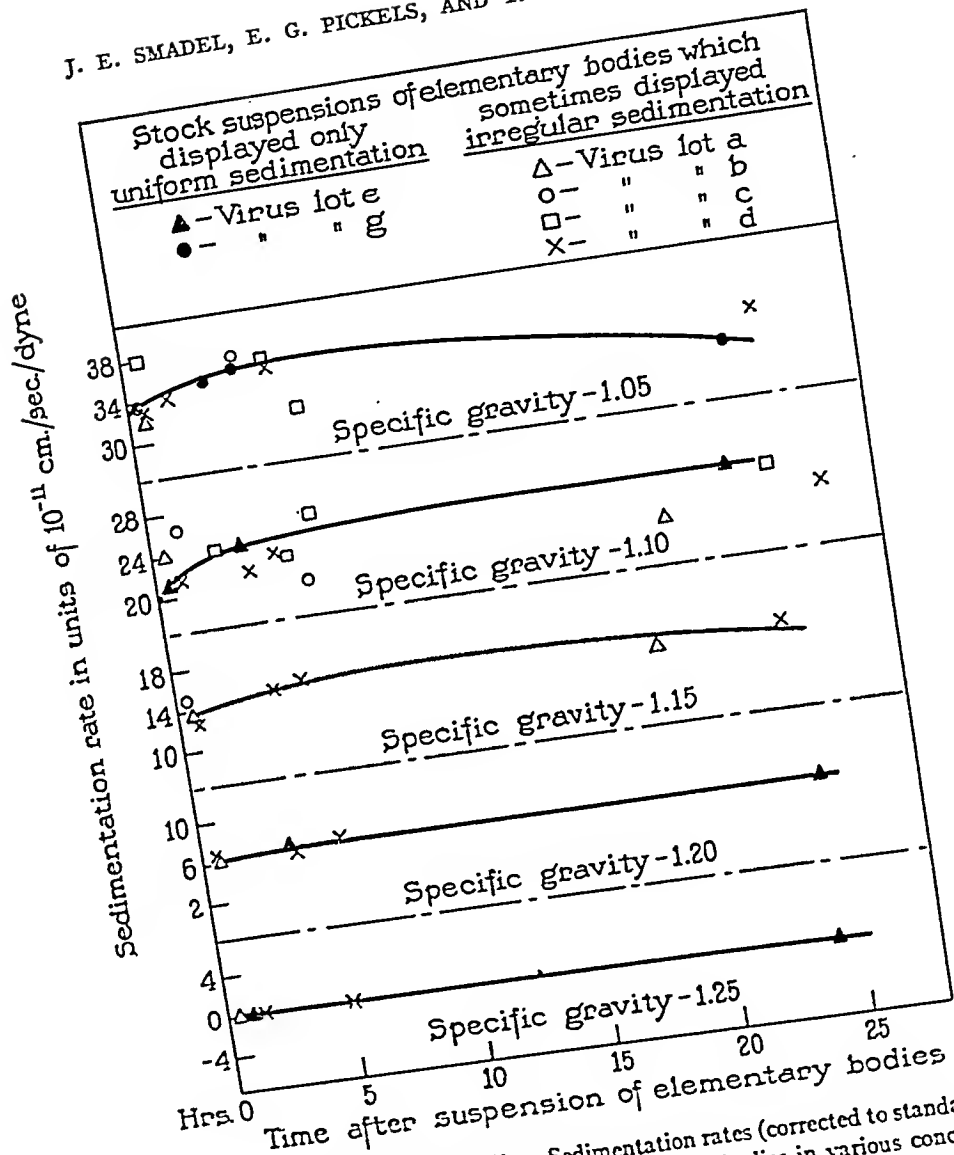
FIG. 2. *Sucrose Suspending Media.*—Sedimentation rates (corrected to standard viscosity) obtained after the suspension of elementary bodies in various concentrations of sucrose. The curves represent the most probable values and are based, for the most part, on measurements made with stock suspensions of elementary bodies which consistently displayed only uniform sedimentation. Irregular sedimentation is discussed in the text, page 610.
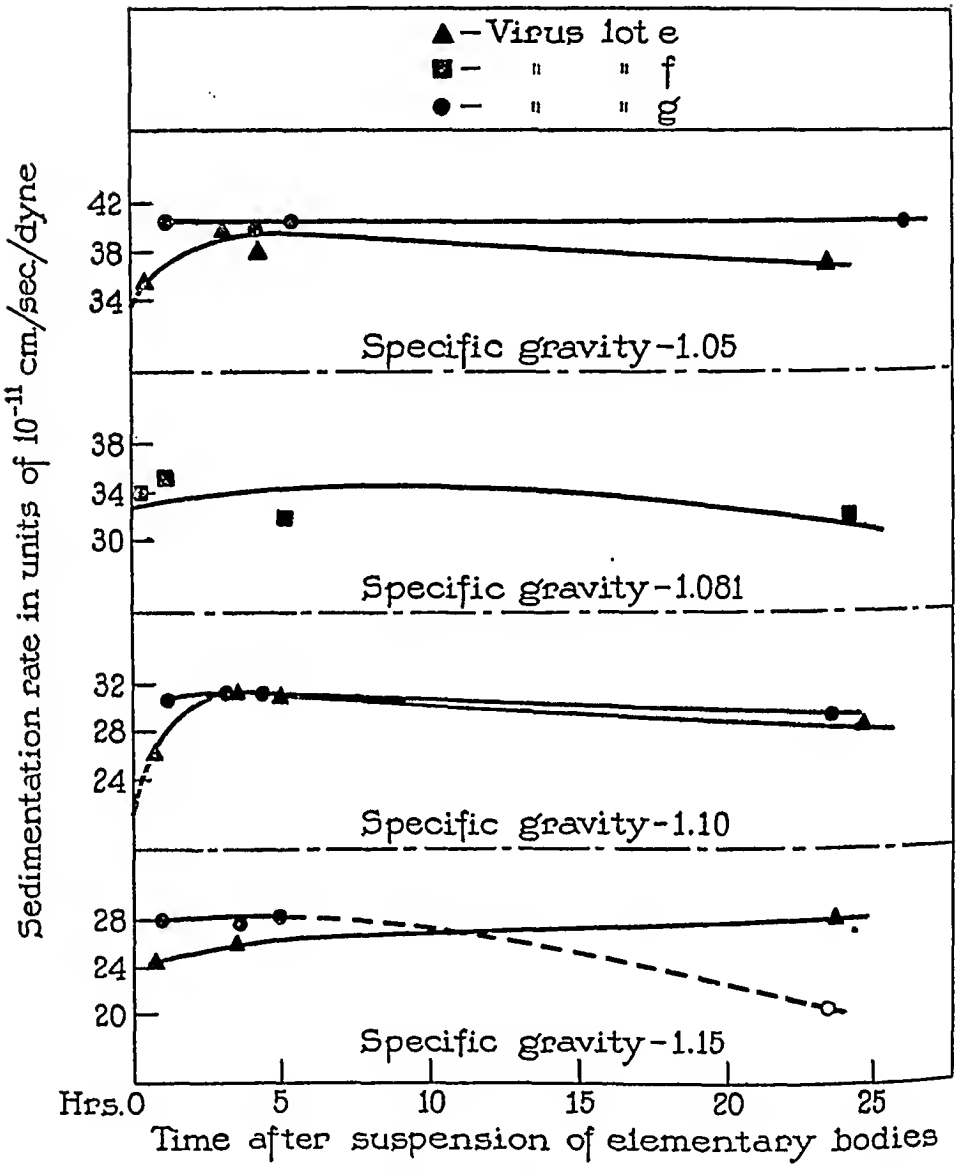
FIG. 3. *Glycerol Suspending Media.*—Sedimentation rates (corrected to standard viscosity) obtained after the suspension of elementary bodies in various concentrations of glycerol. The irregular sedimentation observed with virus lot *g* after 24 hours' contact with glycerol, specific gravity 1.15, is discussed in the text, page 612.

performed during the 5 hours following suspension. After 24 hours this latter virus specimen displayed a very peculiar type of irregular sedimentation, the boundary being sufficiently well defined for measurement during only the middle

portion of the centrifugation. Its sedimentation rate was estimated to be 20.5, a value considerably below the others recorded for the same medium. The details of this experiment have been discussed more thoroughly in another paper (8). It appeared that virus lot *g* might have responded more quickly than lot *e* to the action of the suspending medium, possibly because of some intrinsic
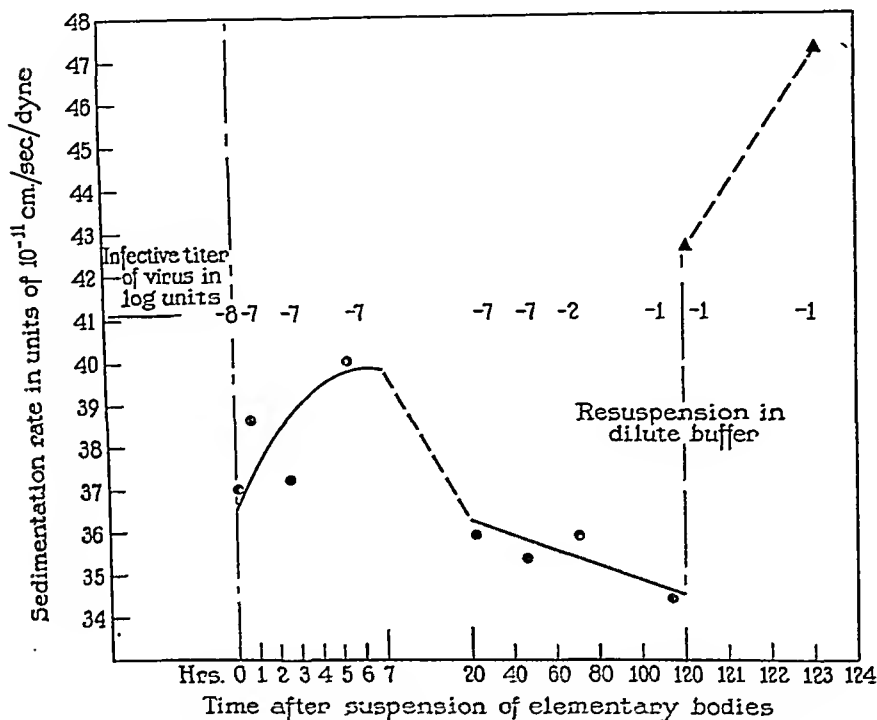
FIG. 4. *Urea Suspending Medium.*—Sedimentation rates (corrected to standard viscosity) obtained after the suspension of elementary bodies in a urea solution of specific gravity 1.06. The virus particles were removed from the urea solution after 120 hours and resuspended in dilute buffer solution. The infective titer of the specimen was determined at the time of each centrifugation. Multiple sedimentation boundaries and a progressively increasing agglutination of virus particles appeared on resuspension.

differences in the elementary bodies. However, the observed discrepancies might possibly have been the result of some slight differences in the preparation, storage, or handling of the specimens of virus. For instance, the effect of temperature on the rapidity and the extent of alteration of elementary bodies by glycerol has not been studied.

The behavior of elementary bodies of vaccinia suspended in urea was studied most thoroughly in solutions of approximately 22 per cent concentration (specific gravity 1.060).   The sedimentation rates determined with a representative specimen of virus particles are plotted graphically in Fig. 4.   The initial rise in the sedimentation rate indicated by the curve was verified by five additional experiments performed with elementary bodies taken from a different stock suspension of virus.   The changes of sedimentation rate in the other solutions of urea represented in Fig. 1 were followed by centrifugation runs over a period of 6 hours. Marked increases in rate were observed during the first few hours of suspension with all the urea solutions except the most concentrated one used (specific gravity 1.100, urea concentration approximately 40 per cent).   The single specimen of elementary bodies studied in this medium sedimented most rapidly during the first experiment, in which the earliest photograph was taken 7 minutes after suspension.   The rate did not appear to be constant throughout the centrifugation, but decreased from an estimated value of 30.6 to about 26.7 between the first and second halves of the experiment.   Over a 4 hour period the rate progressively decreased to a value of 25.5.   It is probable that during the few minutes preceding the first photographs the potential rate had gone through a maximum value and had already begun to decline before measurements could be made. The initial rise and fall of sedimentation rate in a 40 per cent urea solution apparently occurred considerably more rapidly than was observed with 22 per cent urea (Fig. 4).

From the above experiments it appears that the potential sedimentation rate of elementary bodies of vaccinia always increased immediately after their suspension in solutions of sucrose, glycerol, or urea, although it was not always possible to demonstrate such increases in the higher concentrations of these media.   In 22 and 40 per cent urea solutions a marked decline in sedimentation rate followed the initial increase.   Examples of such decreases in rate were also observed with each of four concentrations of glycerol, whereas a distinct lowering of rate was observed with only one specimen of elementary bodies suspended in sucrose solutions, i.e., the specimen from virus lot g suspended in the solution of specific gravity 1.05.   The maximum rates given in Fig. 1 were determined from the maximum heights of the curves given in Figs. 2, 3, and 4 and from similar curves constructed for the results with urea which are not illustrated.   An average value was taken where more than one curve was drawn for a particular medium.

Sedimentation of Elementary Bodies after Resuspension from Solutions of Sucrose, Glycerol, and Urea into Dilute Buffer Solution.—The results presented in previous sections indicate that the physical nature of Paschen bodies is altered when the particles are suspended in solutions of sucrose, glycerol, or urea.   It was considered essential to investigate the possibility of a reversal of these changes.   This was done by measuring the sedimentation rates of elementary bodies that had been removed from such solutions and resuspended in dilute buffer solution.

Virus particles which had been suspended in a 35 per cent sucrose solution for 24 hours were separated from the body of the medium by centrifugation and were then resuspended in 3 cc. of a dilute buffer solution.   2 per cent normal rabbit serum had been incorporated in both media.   The sedimentation rate measured 6 minutes after resuspension was 49.1×10^{-11} cm./sec./dyne; 44 minutes after resuspension it was 48.2.   These values were computed on the assumption that the viscosity of the suspending medium was the same as that of water. Actually, it was slightly higher, since the incorporation of a few drops of sucrose solution in the dilute buffer solution was unavoidable during the resuspension. Nevertheless, the corrected sedimentation rates would still be approximately normal for a dilute buffer solution.   The spread and general appearance of the primary boundaries were normal.   There was no marked agglutination of elementary bodies and there were only faint traces of multiple boundaries.

Elementary bodies of vaccinia which had been suspended for 26 hours in the glycerol medium of specific gravity 1.081 (approximately 34 per cent glycerol) were separated from the medium by centrifugation and then resuspended in 3 cc. of a dilute buffer solution.   Both media contained 2 per cent by volume of normal rabbit serum.   The sedimentation rates were 49.8, 50.6, and 52.2 after 6, 59, and 109 minutes respectively.   Distinct multiple boundaries (see Paper I (8), page 594) were visible in the first and third of these experiments, but not in the second.   The specimen of elementary bodies used for these three experiments had not shown multiple boundaries while suspended in the glycerol solution.

Three experiments were performed on a specimen of elementary bodies after resuspension in a dilute buffer solution from a 22 per cent urea solution in which the particles had remained for 120 hours.   The behavior of this sample is illustrated in Fig. 4.   7 minutes after resuspension the sedimentation rate was only 42.6; in the next 3 hours it rose to 47.2.   This value may be considered as approximately normal for dilute buffer since no correction was made for the slightly higher than normal viscosity of the buffer solution, which resulted from the few drops of urea solution carried over unavoidably during the resuspension.   There were multiple boundaries in both instances, with indications of marked agglutination of the elementary bodies into larger aggregates.   Only about 20 per cent of

the particles were unagglutinated in the first experiment and only about 5 per cent in the second. After 22 hours agglutination was so complete that no distinct boundary was observed on centrifugation.

A specimen of Paschen bodies which had been in contact with a 40 per cent urea solution for 24 hours was resuspended in dilute buffer solution and studied in four experiments. 4 minutes after resuspension the measured sedimentation rate was 44. It increased to 45.6 in the next 3 hours, and to 46.1 after 76 hours. Distinct multiple boundaries were present in the first three experiments. The concentration of the single virus particles constituting the primary boundary was progressively lower in each experiment. Almost all of the particles had agglutinated after 76 hours. Again no correction was made for the slightly higher than normal viscosity of the buffer solution.

To test the possibility that the failure of this last resuspended sample to reach the normal rate of sedimentation in buffer solution might have been the result of some permanent change in the bodies caused by contact with concentrated urea, a sample of virus material was suspended in a dilute urea solution whose concentration was then increased in several steps over a period of about 20 hours to a value of 40 per cent. After remaining for several hours at this concentration the solution was diluted at intervals with a buffer solution until, 48 hours after the original suspension, the concentration of urea had been reduced to 7 per cent. The elementary bodies were then centrifuged from the dilute urea solution and resuspended in buffer solution. A few minutes after resuspension the sedimentation rate was 50.1; 45 minutes later it was 48.5. Thus both values were within the normal range for elementary bodies suspended in dilute buffer solution. Pronounced secondary boundaries of identical appearance were present in both instances (8), but no appreciable agglutination of the particles into large aggregates was observed.

The experiments just described indicate that the changes in the physical nature of the particles which influence the sedimentation rate of elementary bodies placed in contact with concentrated solutions of sucrose, glycerol, or urea are reversible.

The Effect of Different Suspending Media on the Infectivity of Elementary Bodies.—Solutions used in these experiments were chosen not only for their physical properties but also for their relatively mild effect on the infectivity of the virus. The maximum concentration (approximately 59 per cent) of glycerol employed was only a little above that generally used for storing virus-laden tissue, *i.e.*, 50 per cent. The highest concentration of sucrose solution with which centrifugation runs were made was approximately 53 per cent (specific

gravity 1.250). No inactivation of vaccine virus was demonstrated after contact with a 60 per cent sucrose solution for 1 hour.

Urea in certain concentrations, on the other hand, had a deleterious effect on the virus. Elementary bodies suspended for 5 minutes in 40 per cent urea were no longer infective in a dilution of 10^{-1}, whereas before suspension the titer had been 10^{-8}. Inactivation proceeded at a much slower rate when the virus was in contact with 22 per cent urea. The infective titer of the specimen represented in Fig. 4 was obtained from aliquots removed at the time of each of the centrifugations. Titers of the control suspension and of the sample removed after 5 minutes were both 10^{-8}. The five titrations done from 1 to 48 hours after suspension in urea all had the same end point, namely, 10^{-7}. After 72 hours' contact the titer had fallen to 10^{-2}, and at 120 hours to 10^{-1}. Stained smears and studies by means of the dark field revealed no significant alteration in the appearance of the elementary bodies during this period. Fig. 4 indicates the relationship between sedimentation rate and infectivity of the virus. Neither the significant elevation of rate which occurred within the first few hours nor the marked decrease in rate which followed was accompanied by a material drop in infectivity. However, the sedimentation rate continued to decrease slowly after its more rapid initial descent, and it may be significant that the marked reduction in infectivity occurred as the lower sedimentation rates were reached. Inactivation of virus by prolonged contact with high concentrations of urea was irreversible. Elementary bodies after storage for 3 to 5 days in urea solutions of 22 and 40 per cent were sedimented in the angle centrifuge and resuspended in dilute buffer containing rabbit serum. The infectivities of these specimens remained at their previous low levels even after their sedimentation rates became approximately normal. 10 and 15 per cent urea solutions failed to inactivate vaccine virus after contact for as long as 4 days.

The Response of Erythrocytes to Solutions of Sucrose, Glycerol, and Urea.—In order to clarify certain ideas regarding some of the possible ways in which the physical nature of elementary bodies might be altered by suspension of the virus particles in solutions of sucrose, glycerol, or urea, a brief reinvestigation of the response of rabbit

erythrocytes to these solutions was made by means of microscopic observation.

Washed red cells immediately ballooned and burst when placed in physiological saline to which urea had been added in a concentration greater than 5 per cent. Red cells placed in physiological saline containing only 5 per cent urea also swelled, a few sufficiently to hemolyze; the majority, however, returned to their normal size within a few minutes. Such cells, when removed from this medium and placed in physiological saline, first swelled and many hemolyzed; those which did not rupture rapidly shrank, passing through the normal size to become crenated.

Glycerol solutions (1.5 to 50 per cent) in saline failed to hemolyze red blood cells; in fact, the cells appeared to be shrunken in the higher concentrations. Cells which had reached an equilibrium in glycerol solutions of concentrations ranging from 50 to 5 per cent were found to swell rapidly and burst when resuspended in saline. However, practically no hemolysis followed resuspension from a 1.5 per cent glycerol solution.

Sucrose solutions (1 to 50 per cent) in saline produced no hemolysis of red blood cells; on the contrary, the cells became shrunken and crenated. When resuspended in saline, after contact with sucrose solution for half an hour, they returned to normal size. None appeared abnormally swollen and no hemolysis occurred.

These observations agree with the classical experiments on permeability of erythrocytes (10); urea rapidly diffuses into the red cell, while glycerol penetrates it more slowly. Sucrose, on the other hand, does not enter the erythrocyte in appreciable amounts within short periods.

Interpretation of Results

The Density of Elementary Bodies of Vaccinia in Dilute Buffer Solutions.—From the experiments presented above it is apparent that the sedimentation rate of elementary bodies of vaccinia increases, for a time at least, after the particles come into contact with a solution of sucrose, glycerol, or urea. This effect tends to counteract the drop in sedimentation rate which would be expected on transferring the particles from dilute buffer solution to any of these more dense media if the physical condition of the particles were to remain unchanged. A given increase in the density of sucrose solutions produces a greater decrease in the sedimentation rate of the virus particles than does a similar change in the density of glycerol or urea solutions (Fig. 1).

Furthermore a given increase in the density of dilute sucrose solutions changes the sedimentation rate of the particles more than does a similar increase in the density of the more concentrated ones, as is evidenced by the more rapid fall of the curve for sucrose (Fig. 1) at the lower density values. A straight line drawn tangent to the curve for sucrose solutions at a specific gravity value of 1.00 indicates the behavior that elementary bodies would exhibit in a hypothetical medium in which the rate of sedimentation would decrease linearly with increasing density in a manner corresponding to very dilute sucrose solutions. The sedimentation rate in such a medium becomes zero at a specific gravity of 1.17, indicating equal densities for the particles and the medium. This value should represent the maximum possible limit for the density of elementary bodies suspended in dilute buffer solutions.

A more precise determination for the density of the particles in dilute buffer solution would be available if the sedimentation rate of elementary bodies in one of the denser media could have been obtained before any changes in the physical nature of the particles had occurred. The unaltered density of the virus corpuscles could then be computed from the simple mathematical expression relating the sedimentation rate (corrected for the viscosity of medium) directly to the difference between the density of the medium and of the particles:—

$$\sigma = \frac{S_1 \rho_2 - S_2 \rho_1}{S_1 - S_2} \tag{1}$$

in which S_1 and S_2 are the sedimentation rates in dilute buffer and in the denser medium having the respective densities of ρ_1 and ρ_2. Unfortunately such a condition could not be realized experimentally. However, it appeared probable that sedimentation rates for the unaltered particles might be approximated by extrapolating the sedimentation *versus* time curves to zero time. Extrapolated rates were thus obtained from the curves shown in Figs. 2 and 3, and an estimate of the density of elementary bodies in dilute buffer was made with the aid of Equation 1. From the results with sucrose solutions of specific gravities 1.050 and 1.100, density values of 1.158 and 1.165 gm. per cc. were obtained for the virus particles. Densities of 1.156 and 1.167 gm. per cc. were calculated from the extrapolated values for glycerol solutions of specific gravities 1.050 and 1.100. With all the other media investigated, including the urea solutions of low specific gravity, the sedimentation rates apparently were increased so rapidly following suspension that the curves could not be extrapolated with sufficient accuracy to be significant.

On the basis of these extrapolated determinations elementary bodies of vaccinia appear to have a density of approximately 1.16 gm. per cc. when suspended in a dilute buffer solution. In arriving at this value no assumptions were necessary regarding the shape and size of the particles, or regarding the method by which the suspending media altered the physical nature of the virus bodies.

Probable Density of Elementary Bodies in Various Concentrations of Sucrose, Glycerol, and Urea.—The density of Paschen bodies was determined directly only in sucrose solutions of specific gravity 1.250, since in this medium only did the sedimentation rate become zero. It is of interest to estimate, if possible, the probable maximum density of the particles in each of the various media investigated.

For the case of a spherical particle whose volume has decreased by an amount equal to the volume of the extracted water, the following formula was derived, relating the sedimentation rate and the density of the particle in any medium. In the derivation the validity of Stokes' law has been assumed for the shrunken particle.

$$\frac{S_2}{S_1} = \frac{(\sigma_2 - \rho_2)}{(\sigma_2 - 1)^{2/3}(\sigma_1 - 1)^{1/3}} \tag{2}$$

in which S_1 = sedimentation rate of particle in medium of specific gravity 1.00 (dilute buffer).

σ_1 = density of particle in medium of specific gravity 1.00 (dilute buffer).

S_2 = sedimentation rate of particle in medium of specific gravity ρ_2.

σ_2 = density of particle in medium of specific gravity ρ_2.

For the case of a particle whose density is altered by the suspending medium through diffusion, adsorption, or chemical phenomena without appreciably changing the size or shape of the particle, the density and sedimentation rate have the following relationship:—

$$\frac{S_2}{S_1} = \frac{(\sigma_2 - \rho_2)}{(\sigma_1 - \rho_1)} \tag{3}$$

The maximum sedimentation rates represented in Fig. 1 were applied to each of these formulas to compute the density values listed in Table I. It is probable that the actual density in each medium lies between the values calculated by the two methods, if the correct density of elementary bodies in buffer is 1.16 gm. per cc. For the sucrose solutions the values computed on the basis of an extraction of water are probably more nearly correct than those computed on the assumption of a constant particle size.

Nature of the Response Shown by Elementary Bodies to Suspending Media.—The increase in sedimentation rate produced by contact of the elementary bodies with solutions of sucrose, glycerol, or urea might be accounted for in several ways. It may be due to: (*a*) an extraction of water from within the particles; (*b*) a partial replacement of occluded water by the denser solute material; (*c*) a decrease

TABLE I

Probable Limiting Values for the Density of Elementary Bodies of Vaccinia in Various Concentrations of Sucrose, Glycerol, and Urea

Medium	Specific gravity of medium	Calculated density of elementary bodies*	
		Assuming extraction of water and shrinkage of particles	Assuming no alteration in size
		gm. per cc.	*gm. per cc.*
Dilute buffer solution	1.000	1.160	1.160
Sucrose, 13 per cent	1.050	1.173	1.168
" 24 " "	1.100	1.194	1.183
" 35 " "	1.150	1.211	1.205
" 45 " "	1.200	1.230	1.228
" 53 " "	1.250	1.250†	1.250†
Glycerol, 21 per cent	1.050	1.203	1.180
" 33 " "	1.081	1.220	1.194
" 40 " "	1.100	1.232	1.203
" 59 " "	1.150	1.283	1.241
Urea, 9.4 per cent	1.025	1.197	1.175
" 16 " "	1.043	1.218	1.183
" 22 " "	1.060	1.224	1.191
" 40 " "	1.100	1.226	1.200

* Based on measured sedimentation rates.

† Direct experimental determinations.

in size and increase in the average density by the detachment of a bound water layer from the surface of the elementary bodies (11); (*d*) a concentration of sucrose, glycerol, or urea molecules at the surface of the virus corpuscles through the action of some chemical or adsorption phenomenon. Experimental and theoretical considerations indicate that the increases in sedimentation rate are associated

principally with changes in the density of the particles rather than with distortions in their shape.

From the experimental evidence available at the present time it is impossible definitely to accept or reject any of these explanations; however, the discussion will consider those possibilities which seem most likely. The simple osmotic extraction of water from the particles appears to be compatible with the experimental observations made with sucrose solutions as the suspending media. The curves in Fig. 5 were constructed in order to ascertain how closely the changes produced in the virus particles by the various concentrations of sucrose, glycerol, and urea were related to the osmotic pressures of these media. The correlation between the relative orders of the two sets of curves tends to support the suggestion that osmosis was a contributing factor in all cases. The same data indicate, on the other hand, that osmosis alone could hardly account for the total increase in sedimentation rate observed in the glycerol and urea solutions. For example, the density of vaccine virus was found experimentally to be 1.25 gm. per cc. in 53 per cent sucrose solution, while the sedimentation rates determined in glycerol and urea solutions of approximately this same osmotic pressure did not correspond to the values expected for particles having the same density, size, and shape as those in 53 per cent sucrose.

The decline of sedimentation rate, which followed the initial rise and was so noticeable with the higher concentrations of urea, was possibly associated with a diffusion of urea into Paschen bodies, with a resultant swelling. The observation that immediately after resuspension from urea solutions into buffer solutions the elementary bodies sedimented below the normal rate, but approached it later, also suggests that urea had entered the particles. The rise of the rate toward normal may have been associated with an outward diffusion of water and urea molecules.

Regarding the decline of sedimentation rate in the test media and the appearance of multiple boundaries following resuspension of elementary bodies in dilute buffer solution, the behavior of the virus particles in glycerol solutions was intermediate between that observed with sucrose and that with urea. It is, therefore, probable that glycerol is capable of penetrating Paschen bodies to some extent.

McFarlane (11) is of the opinion that a particle of vaccine virus

represents a protein molecule surrounded by an atmosphere of closely bound and oriented water ions.   If, as the present experiments sug-
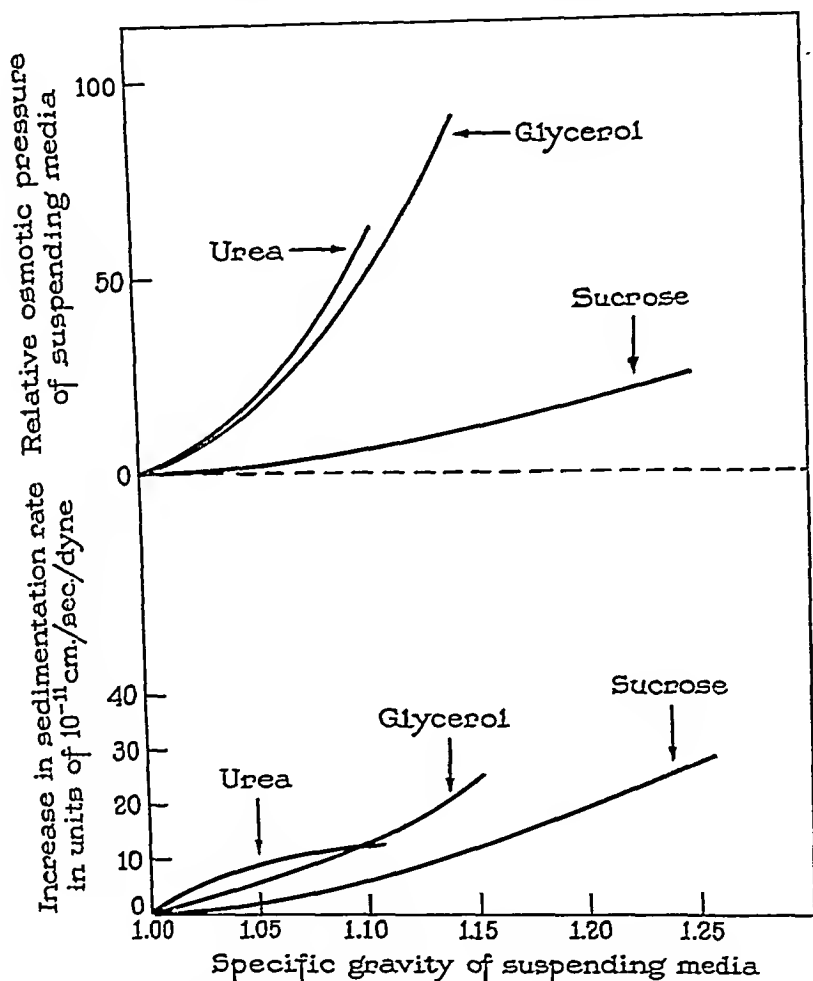
Fig. 5. Increases in the sedimentation rates of elementary bodies produced through the action of the suspending media on the virus particles, compared with the relative osmotic pressures of the respective media.

gest, the extraction of either bound or occluded water accounts for the estimated 8 per cent increase in the density of elementary bodies following their suspension in a sucrose solution of specific gravity 1.25,

there must be a reduction in the particle's effective volume of about 36 per cent. It means that the effective diameter must be reduced by approximately 14 per cent. If. this change in volume is assumed to be due entirely to the removal of a water layer bound to the surface of the particles, it is necessary to postulate a thickness of at least ten water molecules for the layer. Such a particle, suspended in a medium which is essentially aqueous, is difficult to imagine, since the binding forces for the molecules forming all but the inner layers cannot be very different from the forces operating among the free water molecules themselves.

DISCUSSION

Studies on elementary bodies of vaccinia by means of the ultra-centrifuge and the light-absorption method of Svedberg indicate that the virus particles undergo physical changes of a reversible nature when taken from a dilute buffer solution to a solution of sucrose, glycerol, or urea. It appears that sucrose solutions act mainly by removing water from the particle. The effect of urea solution, on the other hand, is apparently more complex; although some water may be removed initially, the urea solution diffuses into the particles within a short time. The action of glycerol probably is similar to that of urea but less marked.

The present estimation of density of the vaccine virus particle (1.16 gm. per cc.) lies between the figure of Elford and Andrewes (4), namely, 1.18 ± 0.02 gm. per cc. and that of MacCallum and Oppenheimer (2), 1.12 to 1.13 gm. per cc. Bechhold and Schlesinger's (3) figure of 1.10 gm. per cc. is considerably lower than our estimate; their figure is close to that recorded for bacteria (12).

It is worthy of note that other authors (4, 13) have clearly recognized the possibility that the density of small biologically active particles might be altered by varying the nature of the suspending medium. Our experiments indicate that the density of elementary bodies generally increases in concentrated solutions. It need hardly be said that the ultracentrifuge combined with the photographic recording of sedimenting boundaries serves as a useful tool in the determination of the size and density of small particles. It may be fallacious, however, to consider the density of the particle in its native state to be the same as that of the suspending medium in which zero

sedimentation occurs. Such an assumption is justifiable only after it has been shown that the physical nature of the given particle is not affected by the concentrated solutions employed.

CONCLUSIONS

Ultracentrifugal studies of the CL dermal strain of vaccine virus warrant the following conclusions:

1. When suspended in increasing concentrations of sucrose, glycerol, or urea solutions, elementary bodies of vaccinia show variations in sedimentation rate which indicate changes in the density or size of the particles. For a given change in the density of the medium these changes are smallest with sucrose and most marked with urea. The normal rate of sedimentation of Paschen bodies may be restored by resuspending them in dilute buffer solution.

2. The density of elementary bodies of vaccinia suspended in dilute buffer solutions is estimated to be 1.16 gm. per cc. Higher values for the density are found if the particles are suspended in solutions containing sucrose, glycerol, or urea. In 53 per cent sucrose, for example, the density is 1.25 gm. per cc.

3. Paschen bodies appear to be quite permeable to water and urea, less so to glycerol, and only slightly, if at all, to sucrose.

4. The increased density of the elementary bodies of vaccinia in sucrose solutions may be accounted for by an osmotic extraction of water from the particles. On this basis the water which can be thus extracted corresponds to at least a third of the original volume of the particles.

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THE LYMPHATIC PATHWAY FROM THE NOSE AND PHARYNX

THE ABSORPTION OF DYES

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Schulz, Warren, and Drinker (1) noted that Type III pneumococci, instilled into the nose of rabbits, could often be found within an hour in the cervical lymph. This observation formed the starting point of our present experiments on the cervical lymphatic pathway.

The lymphatic vessels of the head and neck, like those of the limbs, fall into a superficial and a deep group. The collecting trunks of the superficial vessels accompany the external jugular vein. The tributaries of the deep group flow into the cervical lymph duct, which runs down the neck on the lateral side of the common carotid artery and internal jugular vein. The superficial vessels empty into the main cervical duct, though the level at which this takes place varies in different species; in the cat and monkey it occurs low down in the neck, in the dog not far from the angle of the jaw. The cervical lymph duct on the left side joins the terminal portion of the thoracic duct, just before it opens into the junction of the internal jugular and subclavian veins. On the right side the cervical duct either joins the right lymph duct, or may open independently into the junction of the right subclavian and internal jugular veins. In the present series of experiments we have cannulated the cervical lymph duct low down in the neck, immediately before it joins the venous system.

When the cervical duct is cannulated, one of three things may happen. (*a*) There may be a spontaneous flow of lymph. This is usually the case in the monkey, and presumably, therefore, in man.

(*b*) In the absence of a spontaneous flow, gentle massage of the duct empties its lymph into the cannula, after which the duct fills up again and the procedure may be repeated. (*c*) Occasionally the flow of lymph is negligible. No satisfactory explanation has yet been offered for this occasional "dryness."

If, in an animal whose cervical duct has been cannulated, one instils into the nose a solution of trypan blue or T-1824[1] in physiological saline, the dye quickly passes through the mucous membrane and enters the lymph. It appears in the cannula within 20 to 30 minutes in the monkey, cat, and rabbit, rather longer in the dog, in concentrations which are weak at first but subsequently undergo progressive increase until a fairly constant level is reached.

An experiment of this kind serves a double purpose. It affords a means of investigating the mechanism whereby the living and intact lining of the nose and pharynx permits the rapid passage of these dyes. It also enables one, on subsequent dissection, to obtain a beautifully stained preparation of the entire cervical lymphatic pathway (Figs. 1, 2, and 3). It is a method of delineating a living and functioning system of vessels while actually at work, and is free from the disadvantages of the injection methods usually employed.

Material and Technique

The experiments, summarized in Table I, have been performed on 1 monkey (*Macaca mulatta*), 14 cats, 2 dogs, and 6 rabbits. The dyes employed were trypan blue, in a 2 per cent, 3 per cent, or 5 per cent solution in physiological saline, and T-1824 in 1 per cent or 5 per cent solution. In three experiments Hydrokollag, a fine graphite suspension, was used. The dyes, in the concentrations used, had no irritant action on the living tissues, as tested by dropping in the nose and by subcutaneous injection in the authors.

In most of the experiments a cannula was tied in the trachea and the esophagus ligatured. The trachea was cannulated in order to prevent the dye from entering the lungs and being absorbed into the blood through the pulmonary capillaries,

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[1] The dye T-1824, from a lot prepared by Drs. Hartwell and Fieser of Harvard University in 1936, was furnished us through the great kindness of Professor Magnus I. Gregersen. This sample is free from salt and contaminating isomers. Efforts have been made to call the compound "Evans blue," but to avoid confusion we have preferred the original designation which is also the one used by Gregersen and Gibson (2) in their paper on the behavior of certain vital dyes in plasma.

and in order to block submucous lymphatics. It is of interest that in control experiments in which the trachea and esophagus were not interrupted, dye absorption was equally rapid. All animals were completely anesthetized by nembutal.

The Anatomical Pathway

The superficial lymphatics of the head drain the skin, salivary glands, and the mucous membrane around the buccal margin and nostrils. The deep lymphatics drain the mucous membrane of the mouth, nose, accessory air sinuses, and pharynx. In the cat and dog the deep lymphatics empty into the large superior deep cervical node, close to the bifurcation of the common carotid artery. From the posterior border of this node the cervical duct takes origin, descending to the outer side of the carotid sheath, usually close to it but occasionally situated more laterally. The cervical duct is single as a rule, but it may be double, and occasionally may even be replaced by a plexus of vessels. The duct usually empties on the left side into the thoracic duct, on the right side into the right lymph duct, although many variations are possible.

The cervical lymph duct stands out in our dye experiments as a blue cord, beaded on account of the distension of the segments between the regularly placed valves. It runs down from the posterior border of the superior deep cervical node. Before dye can enter the cervical duct it must first pass through the node, which it therefore colors, usually more deeply at the upper pole than the lower. In the dog one sometimes sees the coloration limited to the posterior portion of the node, with a fairly sharp line of demarcation between this and the unstained anterior portion. In a well marked case this functional segmentation is very striking. In the dog the origin of the cervical duct from the posterior border of the large superior deep cervical node is very evident.

Above the upper pole of the superior deep cervical node, and converging to it, are a number of lymphatic vessels. The largest, two or three in the cat, four or five in the dog, can be traced upwards as far as the pterygoid hamulus, and then between this and the eustachian tube. On removal of the hamulus they can be dissected further as they run forward on the floor and side wall of the nose.

The tonsil was never colored in our experiments, even where the surrounding mucous membrane was obviously stained. One or two small lymph vessels were sometimes seen dorsal to the tonsil or caudal to its lower pole.

All the lymph vessels from the nasopharynx entered the superior deep cervical node. No vessels were seen running down directly into the cervical duct without first of all passing through the node. This accords with the conclusion of Drinker, Field, and Ward (3) on the filtering capacity of lymph nodes, which held that lymph does not return to the circulation without passing through at least one node.

In the cat the cervical duct occasionally passes through an additional node low down in the neck. This occurs even more frequently in the rabbit. It is in the monkey, however, that the interposition of extra nodes along the course of the cervical duct reaches its most advanced degree of development. (Figs. 1,

TABLE I

Summarized Data of Experiments

Experiment No.	Animal	Dye used	Concentration	Amount instilled in nostrils		Time dye placed in nostrils	Time first noted in lymph	Remarks
			per cent	Right cc.	Left cc.			
1	Cat	Trypan blue	Unknown	1.0	1.0	10:00	10:20	
2	"	"	2	0.5	0.5	3:15	3:40	
3	"	"	5	0.5	0.5	3:10	4:20	
4	"	"	3	None	1.0	2:45	3:00	Duct not cannulated; found only after being colored by dye
5	"	"	3	1.0	1.0	10:30		Cervical ducts not cannulated. Killed at 2:05 for examination of interior of cranium. No blue found
6	Dog	"	3	2.0	2.0	2:04	2:55	
7	Cat	T-1824	5	1.5	1.5	2:40	3:00	
8	"	"	5	0.5	1.0	12:15	12:37	
9	"	"	5	1.0	1.0	11:30	11:55	
10	"	"	5	2.0	—	10:05	10:10	
11	Dog	"	5	1.0	1.0	11:57	12:50	Dye observed in afferent vessels to superior deep cervical node
12	Rabbit	"	5	1.0	—	1:45	1:59	
13	"	"	5	1.5	1.5			Left in nose 2 hrs. and 35 min. Cranium then opened and interior of skull examined, particularly region above cribriform plate. No dye found. (See Table II)‡
14	"	"	5	1.5	1.5			Left in nose 2 hrs. and 55 min. Head examined as in No. 13. Possibly a faint trace of dye found in one olfactory lobe
15	"	"	5	1.0	1.0			Nos. 15, 16, and 17 unanesthetized rabbits in which dye was dropped into the nose. There was no sneezing, coughing, or any other sign of irritant action of the dye. The animals were killed after 1½ hrs., and the cervical pathway was fully stained
16	"	"	5	1.0	1.0			
17	"	"	5	1.0	1.0			

18	Monkey	T-1824	5	1.5	1.5	12:45	1:10	
19	Cat	Hydrokollag		1.0	1.0	10:37	Not found	Hydrokollag left in nasopharynx for 6 hrs. Heads then placed intact in fixative,
20	"	"		1.0	1.0			and neck dissected out 1 month later. No trace of Hydrokollag in lymph
21	"	"		1.0	1.0			nodes or in cervical duct
22	"	"		2.0	2.0			Hydrokollag left in nasopharynx for 6 hrs. Neck then dissected, and no trace of Hydrokollag found in cervical node or duct
23	"	"		1.0	1.0	1:00	Not found	

2, and 3.) Here the duct constantly passes through a chain of five or six nodes, and it is noteworthy that the entire duct passes through each node, not merely a tributary.

The Passage through the Mucous Membrane

Practically nothing is known of the mechanism whereby animate or inanimate particles pass through the mucous membrane and enter the lymphatic vessels. There is some evidence, however, that the size of the particles is an important factor. In our experiments trypan blue (molecular weight 960.81) and T-1824 (molecular weight 960.81) passed through readily. Hydrokollag, a fine graphite suspension with particles ranging in size from 0.2 to 2.0 micra, did not reach the lymph. Data concerning experiments with proteins and viruses will be presented in other papers.

The Appearance of Dye in the Lymph

Following the instillation of dye into the nose, the cervical lymph after a short period shows a faint tinge of blue. Subsequently the concentration of dye gradually increases. The time which elapses between the placing of the dye in the nose and its appearance in the cannula is 15 to 30 minutes in the cat and monkey, nearer an hour in the dog (Table I). In either case, however, the time is considerably in excess of that required for the dye to pass through the nasal mucosa. For after its passage through the mucosa the dye has to traverse the large superior deep cervical node, and almost the entire length of the cervical lymph duct, before it appears in the cannula. In other words, the dye-containing lymph has to displace the normal lymph previously present in the node and vessels before its presence in the cannula can be detected. If, however, one dissects high up in the neck, above the superior deep cervical node, one can observe the lymph vessels close to their commencement in the nasopharynx, and the appearance of dye in them may be noted in a very much shorter time. This is illustrated by Experiment 10.

Experiment 10. Dissection of Afferent Vessels to Superior Deep Cervical Node.— Cat, weight 3.5 kg. Feb. 11, 1938. *9:00 a.m.*, general anesthesia by nembutal (5 per cent) 4.0 cc. intraperitoneally.

10:05, dissection of superior deep cervical node and afferent lymph vessels complete on right side. 2.0 cc. of 5 per cent T-1824 in physiological saline placed in right nostril.

10:10, dye observed streaming down in afferents to node.   Progressive coloring of node from above downwards, finally reaching efferent lymph in the cervical duct after 20 minutes.

Note.—The passage through the mucosa in so short a time seems to indicate that the question of deterioration of the mucosa does not arise.   Further, no tracheal cannula was used in this experiment, nor was the esophagus ligated, so that the condition of the nasal mucosa was presumably normal.

The changing concentration of dye in the cervical lymph is best shown by taking samples of lymph at intervals in fine capillary tubes of equal bore.   Fig. 4 depicts a set of such tubes (Experiment 18). Dye was placed in the nose of a monkey at 12:45 p.m. and appeared in the cannula at 1:10 p.m.   After an initial period of rapid increase, the concentration of dye becomes much more stable.   The last few samples of lymph show very little change.

The concentration of dye can be estimated with fair accuracy by comparing the set of capillary tubes with another set of tubes of the same internal diameter containing a series of known dilutions of the dye (Fig. 5).   This is illustrated by the protocol of the experiment from which the set of tubes in Fig. 4 were obtained (Experiment 18).

Experiment 18.—Monkey (*Macaca mulatta*), weight 3.5 kg.   Feb. 15, 1938. *9:20 a.m.*, general anesthesia by nembutal (5 per cent) 3.5 cc. intraperitoneally. *10:20*, trachea cannulated.   *11:55*, right cervical lymph duct cannulated.   Protein 3.65 per cent.

12:15 p.m., blood sample 1 taken.

12:45–12:48, T-1824 (5 per cent in physiological saline) 1.5 cc. in right nostril. *12:48–12:55*, equal amount in left nostril.   *12:50*, lymph flowing spontaneously. Small amount of dry heparin placed in the cannula after withdrawal of each lymph sample, to prevent clotting.   *1:05*, lymph flowing well.   *1:50*, lymph still flowing spontaneously, but rate of flow slower.   *2:45*, rectal temperature 98°C.

2:47, blood sample 2 taken.

2:50, animal killed by bleeding and injection of 5.0 cc. of nembutal (5 per cent) intravenously.

Concentrations of Dye in Lymph

Time	Concentration	Time	Concentration
1:20 p.m.	1:128,000	2:10 p.m.	1:4,000
1:30	1:32,000	2:20	1:3,000
1:40	1:16,000	2:30	1:2,000
1:50	1:8,000	2:45	1:2,000
2:00	1:8,000		

Lymph flowed for 15 minutes after death, and a sample taken at 3.00 p.m. had a dye concentration of 1:1,000.

Concentration of Dye in Blood.—
Blood sample 1.... No dye.
Blood sample 2.... Less than 1:128,000.
Protein in Blood Serum.—5.83 per cent.

Absorption of Dyes Directly into the Blood Stream

Some of the dye placed in the nose is absorbed directly into the blood stream. This can readily be shown by ligaturing the superficial and cannulating the deep lymphatics in the neck, and taking repeated blood samples as has been done in Experiment 9.

Experiment 9.—Cat, weight 3.5 kg. Jan. 20, 1938. *9:25 a.m.*, general anesthesia by nembutal (5 per cent) 3.5 cc. intraperitoneally. *9:50*, trachea cannulated, esophagus ligated. *10:00*, right external jugular lymph vessels ligated. *10:25*, right cervical lymph duct cannulated. Moderate flow. *11:05*, left external jugular lymphatics ligated. Left cervical duct cannulated. Flow slight. *11:25*, lymph now flowing spontaneously on both sides. *11:28*, nembutal 1.0 cc. intraperitoneally.

11:30–11:40, T-1824 (5 per cent in physiological saline) 1.0 cc. in each nostril.

11:42, protein in right cervical lymph, 3.33 per cent. Spontaneous flow. *11:48*, protein in left cervical lymph, 3.26 per cent. Flow spontaneous also. No massage employed as yet.

11:55, right cervical lymph blue in cannula. *11:57*, nembutal 1.0 cc. intraperitoneally.

12:00, left cervical lymph also now visibly blue in cannula.

12:20 p.m., lymph hardly flowing on left side. No massage employed. Still flowing spontaneously on right.

1:30, right cervical lymph still flowing spontaneously, but on left side lymph now obtainable only on duct massage. *2:00*, flow of right cervical lymph almost ceased. *2:40*, animal bled to death.

Post Mortem.—Right. External jugular lymphatics dilated with colorless lymph. Superficial cervical lymph nodes hardly stained. On deep dissection the superior deep cervical node was deeply stained in its upper three-fourths. Only one efferent vessel found, the one cannulated. No sign of any other lymph vessel.

Left. External jugular lymphatics dilated up to site of ligature and contain pale blue lymph. Superficial lymphatics from angle of mouth coursing over masseter also blue. Superficial cervical lymph nodes stained, though not heavily.

Superior deep cervical node deeply stained. No bypassing by any other vessel.
No inferior node. Marked extravasation of lymph along course of duct.
Cranium. Cribriform plate exposed from above. No sign of blue.

Concentrations of Dye in Blood and Lymph

Time	Blood	Lymph
11:20 a.m.	Normal	Normal
12:10 p.m.	1:128,000	1:4,000
12:40	1:100,000	1:500
1:10	1:64,000	1:250
1:40	1:32,000	1:250
2:10	1:16,000	1:250
2:40	1:16,000	1:250

The blood at 2:40 p.m. had slightly more dye than at 2:10, but the difference
was not sufficiently marked to be measured by our scale.

It will be noted that in both the blood and the lymph the concentration of dye
tends to reach a constant level. There is, however, the important difference that
the concentration in lymph is very much higher than that in blood.

Communications between the Nose and the Interior of the Cranium

Several observers have described the rapid passage of simple solutions from
the nose into the cranial cavity. Clark (4) states that:

"A solution of potassium ferrocyanide and iron ammonium citrate, dropped into the
nasal cavities of rabbits, reaches the surface of the brain within one hour." He believed
that there was a pathway along "the perineural sheaths of the olfactory nerves," and
concluded that "the spaces of these perineural sheaths are continuous above with the
subarachnoid spaces and extend peripherally along the peripheral fibres of the olfactory
nerves to the olfactory sensory epithelium. The existence of a current running centrip-
etally in these perineural sheath spaces under normal conditions is postulated."

Faber (5) came to similar conclusions as a result of experiments on rabbits.
These apparently conclusive findings for simple crystalloids have not been con-
firmed by our experience with trypan blue and T-1824. In but one instance have
we seen the slightest trace of dye in one olfactory lobe, and in that case the
appearance was so slight as to be a matter for dispute in the laboratory.

In regard to visible particles, Clark (4) found that particles of carbon failed
to reach the inside of the skull after nasal instillation. Our experience with
Hydrokollag was similar. On the other hand, Olitsky and Cox (6), in mice,
found that Prussian blue granules did pass from the nose into the cranium.
Rake (7) made the same observation in mice, and also found that pneumococci
and *Salmonella enteritidis* reach the subarachnoid space "with the same rapidity
as the pigment," as also does the pantropic virus of equine encephalomyelitis,
although neurotropic viruses were not demonstrated in less than 24 hours.

"That the lymphatics of the nasal mucosa are in almost direct communication with the subarachnoid space has been clearly demonstrated" (Peabody, Draper, and Dochez, 8). This statement, however, can only be made with certain reservations. It is true that substances introduced into the subarachnoid space escape into the

TABLE II

Passage of Substances from the Nasal Cavity through the Cribriform Plate

Experiment No. (See Table I)	Animal	Material placed in nose	Time left in nose
5	Cat	3 per cent trypan blue	3 hrs. and 35 min.
7	"	T-1824 (5 per cent)	2 hrs. and 35 min.
9	"	" " " "	3 hrs. and 10 min.
9a	"	" " " "	5 hrs. and 50 min.
11	Dog	" " " "	3 hrs.
13	Rabbit	" " " "	2 hrs. and 35 min.
14	"	" " " "	2 hrs. and 55 min.
23	Cat	Hydrokollag	6 hrs.

No dye was found above the cribriform plate in any experiment except possibly Experiment 13. In the rabbit, even where dye is not found above the plate, the plate appears blue on looking down from above. Either the plate is so translucent that one can see the dye in the roof of the nose through it, or the dye may have stained part of it.

lymphatics of the nasal mucosa and then into the cervical lymphatics. Since the first observations of Key and Retzius (9) many workers have confirmed this fact. (For full bibliography see Weed, 10.) The question now at issue is whether the reverse proposition is also true, that substances in and upon the nasopharyngeal mucosa which enter cervical lymph may also pass into the subarachnoid space. In the present series of experiments this was definitely not the case. Our results are of especial interest since by cannulation of the cervical lymph duct it was possible to show that dye was present in the lymph in high concentration over many hours, and yet could never be detected in the interior of the cranium (Table II).

Our results therefore appear to conflict with those of previous workers. There are two factors to consider: (a) the nature of the solution used, and (b) the size of the animal. Clark (4) and Faber

(5) both used in their successful experiments simple solutions, potassium ferrocyanide and iron ammonium citrate, whereas in our own experiments colloidal dyes were used.   It is to be noted, however, that Faber, when using thorotrast (a solution of thorium dioxide in a protective colloid),obtained results as completely negative as our own. "The sections from the rabbit into the nostrils of which thorotrast had been instilled were completely negative for the passage of thorotrast through the nasal mucosa." Similarly Clark's results when using trypan blue are in agreement with those now presented.   Clark left a 0.5 per cent solution of trypan blue in the nose of a rabbit for 24 hours, and at the end of that time found no sign of dye within the cranium.   It seems evident that for anything but solutions of simple crystalloids the cribriform plate offers an effective barrier to the passage of substances (non-living) from the nose to the interior of the skull.

Another factor which has been insufficiently considered is the size of the animal.   In a small animal such as the mouse the cribriform plate is an exceedingly tenuous structure, and might conceivably permit a passage of dyes to which the thicker cribriform plate of larger animals would be more resistant.   The rabbit, on the other hand, is much nearer in size to the cat, in which our findings were all negative. In 4 rabbits we instilled T-1824 (5 per cent) into the nose and examined the region of the cribriform plate 3 hours later.   In none was there any real staining above the cribriform plate though in one there may have been a very faint trace of blue in one olfactory bulb.   Our results suggest, therefore, the advisability of interpreting with great caution the results of experiments on small animals.   They may be very suggestive, but at the same time not applicable with certainty to man.

SUMMARY

1. In the monkey, dog, cat, and rabbit the cervical lymph duct was cannulated, and then a solution of T-1824, or trypan blue, or a fine graphite suspension, all in physiological saline, was dropped into the nose.   T-1824 was used in all four animals, trypan blue in the cat and dog, the graphite suspension (Hydrokollag) in the cat alone.

2. No Hydrokollag was ever found in the cervical lymph.

3. Trypan blue and T-1824 appear in the cervical lymph 15 to 30 minutes after being placed in the nose of the cat and monkey, 51 to 53 minutes in the dog, and 14 minutes in the rabbit.

4. T-1824 and trypan blue were also absorbed from the nose directly into the blood.

5. Neither the dyes nor the Hydrokollag, though left in the nose for as long as 6 hours, were found to pass through the cribriform plate and reach the interior of the cranium.

6. In the monkey cervical lymph passes through a chain of five or more lymph nodes, in the rabbit frequently through two nodes, in the cat through one node except in rare instances, and in the dog through one node.

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EXPLANATION OF PLATES

PLATE 25

FIG. 1. Cervical lymphatic pathway in the cat (upper drawing), and in the dog (lower drawing).

Key to lettering in Figs. 1, 2, and 3: *A*, cervical lymph duct; *B*, superior deep cervical node; *C*, afferent vessels to superior deep cervical node; *D*, tonsil; *E*, patches of mucous membrane through which dye can be seen in mouth and pharynx; *F*, superficial cervical node or nodes; *G*, lymph vessels draining from superficial to deep cervical nodes; *H*, chain of deep cervical nodes; *I*, superficial lymph vessel running from cheek and angle of mouth, over surface of masseter, into superficial cervical node; *J*, cannula in cervical lymph duct.
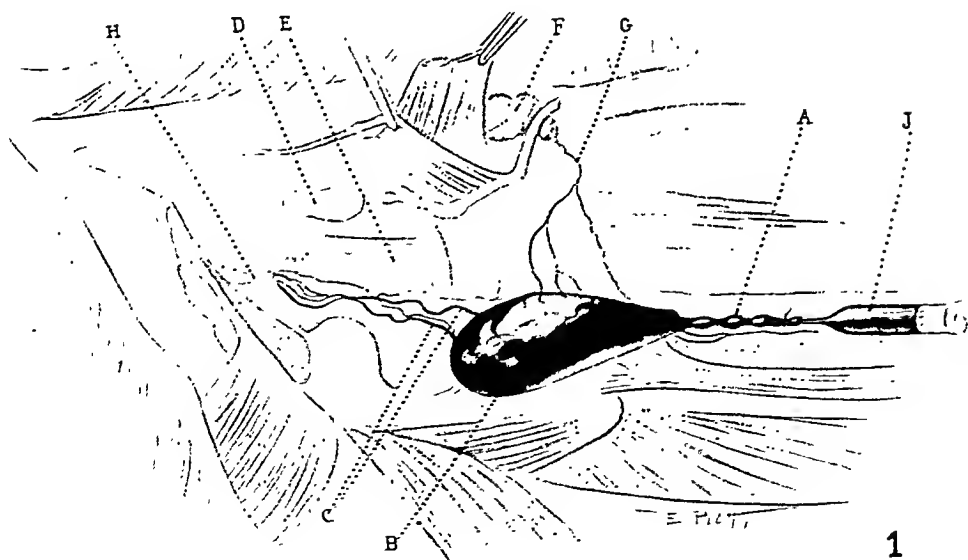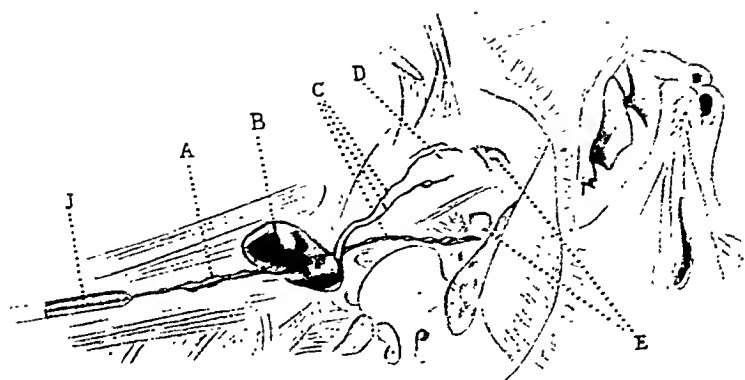
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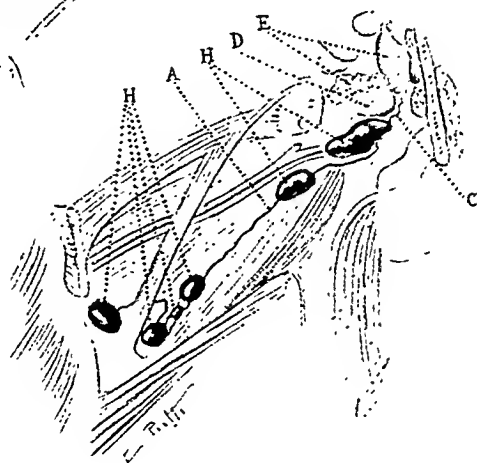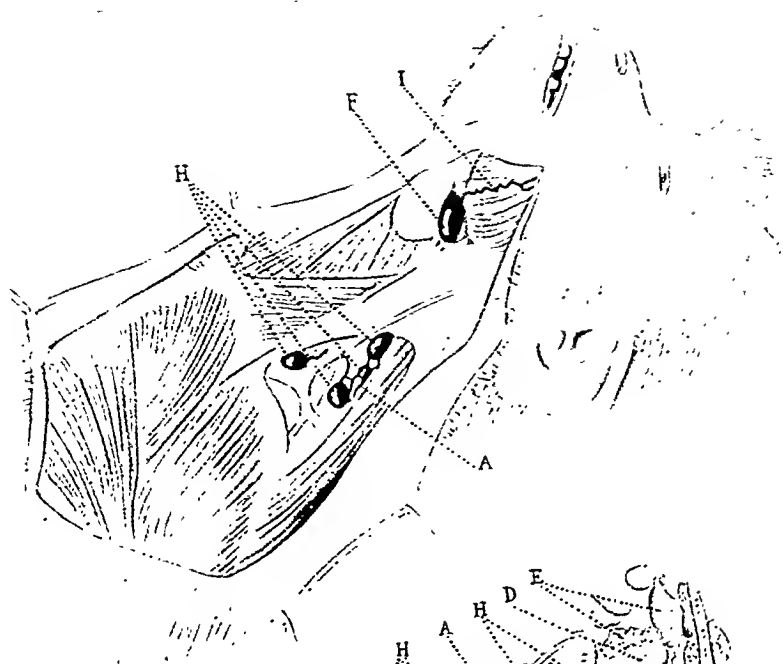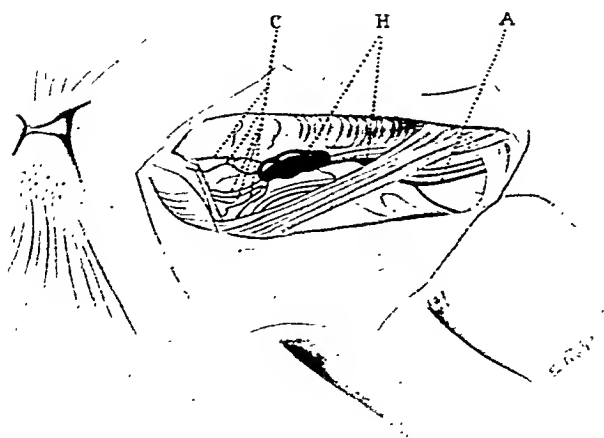
FIG. 4. Set of capillary tubes containing cervical lymph taken at intervals. 1.5 cc. of T-1824 (5 per cent) were placed in the right nostril at 12:45 p.m. It will be observed on looking at the chart that the first appearance of blue in the lymph is at 1:10 p.m. The time at which each sample was taken is given at the side of the tube

FIG. 5. Set of capillary tubes with known dilutions of T-1824.

	12:50 p.m.
	1:00
	1:10
	1:20
	1:30
	1:40
	1:50
	2:00
	2:10
	2:20
	2:30
	2:45
4	Post mortem

	1:128,000
	1:64,000
	1:32,000
	1:16,000
	1:8,000
	1:4,000
	1:2,000
	1:1,000
	1:500
	1:20

5

(Yoffey and Drinker: Lymphatic pathway from nose and pharynx)
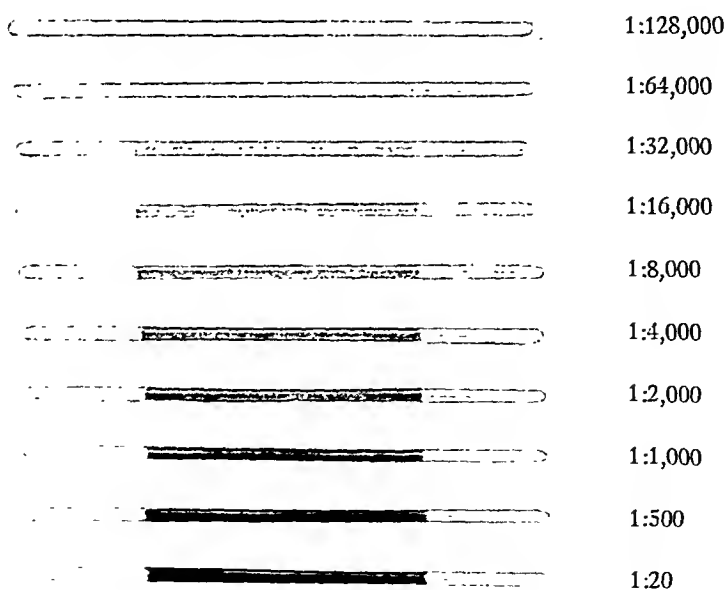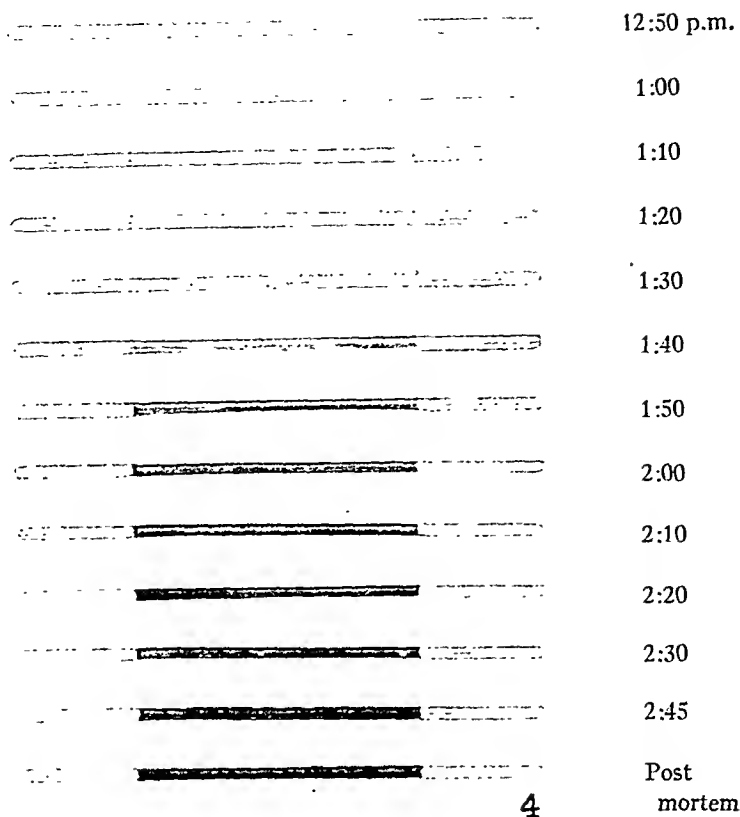
CORRECTIONS

Vol. 68, No. 2, August 1, 1938

Page 223, Table I, last column, 2nd line under per cent, for 7.07 read 70.7.

Vol. 68, No. 4, October 1, 1938

Page 474, 8th line from the bottom, for Ca read G.

REACTIONS OF NORMAL AND TUBERCULOUS ANIMALS TO TUBERCULO-PROTEIN AND TUBERCULO-PHOSPHATIDE

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PLATE 28

In a previous report (1) it was shown that the intracutaneous injection of tuberculo-phosphatide into tuberculous animals produced a local reaction without macroscopic necrosis which persisted for some time. The reaction more closely resembled the Koch phenomenon (altered and accelerated reaction of a tuberculous animal to reinoculation) than a tuberculin reaction. Therefore it was decided to study the cytology of this reaction and compare it with the reaction of tuberculous animals to tuberculo-protein (water-soluble MA-100, Mulford).

Many of the lipid components of tubercle bacilli have been studied by Sabin and her associates (1–10) with reference to the cellular responses of animals to injections of them. The waxes (10) and the acetone-soluble fat (9) are remarkable cell stimulants and each plays a part in producing the cellular picture of tuberculosis. But the reactions to the phosphatide have been the subject of more extensive research because: (a) the phospholipid forms a fine suspension in water whereas the other lipids must be suspended in other media; (b) it induces the formation of tubercular tissue, particularly of epithelioid cells, as shown by Sabin and Doan (2–4) and Sabin, Doan, and Forkner (5–7); and (c) because of the specificity of the reaction induced by it. That is, following the initial reaction of granulocytes, the only cells remarkably affected are monocytes, and the latter are so altered as to become typical epithelioid cells (7, 1).

In all of the studies mentioned above the materials employed have been isolated from tubercle bacilli by Dr. R. J. Anderson[1] whose studies were summarized in 1932 (11). The first specimens of tuberculo-phosphatide prepared by Dr. Anderson contained acid-fast debris and a few tubercle bacilli, to which some of the reaction might have been attributed. More recently he has filtered his phos-

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[1] Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut.

phatide through Chamberland porcelain filters; and the specimens used in the present study, with one exception, failed to show even traces of acid-fast material, or of anything having bacillary form. This fact is mentioned because the belief has been held by some (12, 13) that the capacity of the Anderson phospholipid to produce tubercular tissue was due to the presence of impurities, specifically of acid-fast bacteria.

Boissevain (12, 13) found that a crude phosphatide containing acid-fast bacteria and possessing the capacity to produce hypersensitiveness induced the formation of tubercular tissue, whereas a purified preparation which failed to sensitize also failed to produce tubercular tissue. Later Boissevain (14) expressed the view that the principle which causes hypersensitiveness and tubercle formation is an insoluble protein. Shortly afterward, however, it was indicated (15) that an insoluble compound of soluble protein and soluble polysaccharide fulfilled four properties of an active principle of the tubercle bacillus: tuberculin activity, capacity to form precipitate in immune serum, to induce the formation of tubercular tissue, and to induce hypersensitiveness.

More recently Holley (16) studied the cellular reactions in the cornea of normal and tuberculous guinea pigs after intracorneal injections of tuberculo-protein and tuberculo-phosphatide. He found that the tuberculo-protein produced an acute reaction with the presence of epithelioid cells in the later stages; the phosphatide also produced an acute reaction with epithelioid cells, and these persisted for longer periods than those appearing as a response to protein. The phosphatide which Holley used was bacteria-free, having been filtered through a Seitz disc. But it contained 0.229 per cent nitrogen and he concluded that the reaction induced was due to the presence of an impurity, probably tuberculo-protein firmly bound into the phosphatide molecule. The protein used by Holley was the TPT fraction isolated from the culture medium by Dr. F. B. Seibert.

The experiments which follow indicate that there are both quantitative and qualitative differences between the reaction to tuberculo-protein and that to tuberculo-phosphatide, and that these differences are accentuated in tuberculous animals. Moreover, despite the fact that we have previously shown that the phosphatide does not induce hypersensitiveness to tuberculo-protein, it will be shown that a tuberculous infection does alter the reacting capacity of the animal to an injection of tuberculo-phosphatide.

Materials and Methods

Tuberculo-Protein.—The tuberculo-protein used was the water-soluble MA-100 from human tubercle bacilli.[2] Prior to any other experimental procedure, each

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[2] Prepared by the Mulford Laboratories of Sharp and Dohme, Glenolden, Pennsylvania, and supplied by them through the courtesy of Dr. John Reichel.

tuberculous animal was tested for hypersensitiveness with 0.1 mg. of this protein intradermally. Although there were quantitative differences, each animal exhibited a positive reaction.

Tuberculo-Phosphatide.—The phospholipid used in the first experiment was a lot prepared[3] according to the method of Anderson. The lots of phosphatide used in subsequent experiments were highly purified preparations made by Dr. Anderson to whom we are indebted for them. The preparation used in the final experiment was a crude one made by Dr. Anderson.

Animals.—The animals were young, healthy guinea pigs purchased in the open market. They were carefully selected and observed several days for evidence of intercurrent disease before experiments were begun.

Cultures.—The tuberculous animals were inoculated subcutaneously with 0.01 mg. human tubercle bacilli, strain H-37. In the first experiment the organisms were grown on Petroff's gentian violet-egg medium; in the second and third experiments on Corper's glycerolated egg yolk medium.

Macroscopic Examinations.—Areas of reaction in the skins of normal and tuberculous animals were examined daily and measured with calipers in order to determine quantitatively the extent of reaction.

Cellular Studies.—At the desired time after injection of either tuberculoprotein or tuberculo-phosphatide the animals were sacrificed. At autopsy preparations of the cellular reaction were made for study by the supravital method. Paraffin sections were prepared in the usual manner. Surveys of the visceral organs were made to determine the extent of visceral tuberculosis and to discover any intercurrent disease. .

Reactions to Tuberculo-Phosphatide

Experiment 1.—In the first experiment five normal guinea pigs and five which had been inoculated intradermally in the midline of the lower abdomen with 0.01 mg. of human tubercle bacilli, strain H-37, 49 days previously, were each injected subcutaneously in the right scapular region with 40 mg. of the Mulford phosphatide suspended in 2 cc. of sterile isotonic saline. This preparation was preponderantly non-acid-fast, but some amorphous acid-fast material was present. No tubercle bacilli were found in the stained smears. The macroscopic area of reaction in both normal and tuberculous animals was measured daily; one normal and one tuberculous animal were sacrificed on the 1st, 3rd, 5th, 7th, and 14th days for cytologic studies.

As soon as 24 hours after injection there were marked differences between the reactions in normal and tuberculous animals. No animal exhibited a reaction with necrosis, but erythema, edema, and induration were far greater in the tuberculous than in the normal animals.

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[3] Prepared by the Mulford Laboratories.

At 48 hours the reactions in the tuberculous animals had reached their maximum size and three of four exhibited necrosis at the center of the nodule. After the 2nd day the reactions in the tuberculous animals decreased gradually in size but at the end of a week were still more than twice the size of those seen in the normal animals, as shown in Table I. The area of reaction in normal animals reached its maximum size in 72 hours, then declined gradually, at all times being smaller than the reactions in tuberculous animals, and at no time exhibited macroscopic necrosis.

Table I shows the greater, more accelerated, and more persistent reaction in the tuberculous animals.

TABLE I

Area of Reaction to Phosphatide in Normal and Tuberculous Animals at Various Periods after Injection

Time after injection	Tuberculous		Normal	
	Mean area	Number of animals	Mean area	Number of animals
days	*sq. cm.*		*sq. cm.*	
2	16.77	4	6.89	4
3	12.56	4	10.37	4
4	10.17	3	7.09	3
5	8.84	3	4.27	3
7	7.59	2	3.66	2

Cytologic studies made 24 hours after the injection showed a much larger reaction in the tuberculous animal (measured in sections). Supravital preparations revealed that the reactions were similar in type but characterized by greater relative reaction of polymorphonuclears in the tuberculous, and in these animals the granulocytes appeared damaged, showing many-lobed nuclei and vacuolated cytoplasm. In both normal and tuberculous animals the reaction was characterized principally by polymorphonuclears and monocytes, with small numbers of lymphocytes. Other points of difference at this time were: active cell division in the tuberculous animal and tiny foci of hemorrhage, as seen in sections, together with greater outpouring of granulocytes. Myelin-like masses of free phosphatide were seen in both animals, and some phosphatide could also be seen in mononuclear phagocytes.

At 3 days the reaction in the tuberculous animal exhibited extensive necrosis, as seen in supravital preparations and fixed tissues. There was beginning ulcera-

tion in the skin. Necrosis was minimal in the normal animal. There was a greater monocytic reaction in the tuberculous animal, and less of the phosphatide remained free, but the material was the more easily identified in the cells of the normal animal because they were better preserved. The phosphatide could be recognized in stimulated monocytes or first stage epithelioid cells (1) by the intracellular location of the myelin-like figures. In the tuberculous animal there was evidence also of a fresh outpouring of granular leucocytes, but not in the normal animal. In the latter there were rather few granulocytes but many monocytes and early epithelioid cells, with many of the latter in division. Both animals exhibited evidence of degenerative change in the carnosus muscle.

By the 5th day the granulocytes had almost entirely disappeared in the normal animal and the reaction was characterized almost wholly by monocytes and first and second stage epithelioid cells; a few of the latter were found in the regional lymph node. In the region adjacent to the carnosus were many fibroblasts and a small focus of necrosis. In the tuberculous animal there were still a considerable amount of necrosis and numerous polymorphonuclears. Monocytes and epithelioid cells were also numerous but their nuclei often appeared to be pyknotic. The regional lymph nodes in both animals showed stimulated monocytes and an occasional eosinophile. In the tuberculous animal the skin had ulcerated, there was much edema in the area of reaction, there was still some hemorrhage in the skin, and the whole reaction was much greater than in the normal animal.

After 7 days the reaction in the normal animal was characterized principally by epithelioid cells of the first and second stage (large or medium sized vacuoles) and monocytes, with sparsely scattered neutrophiles and eosinophiles. There were sheets of fibroblasts adjacent to the damaged, regenerating carnosus. Necrosis was minimal. The larger reaction in the tuberculous animal showed that the epithelioid cells had proceeded farther toward dispersion of the phosphatide: some had proceeded to the third or fine vacuole stage. The animal showed necrosis still, greater numbers of neutrophiles than the normal, more degenerative change in the carnosus, and greater numbers of fibroblasts. A few Langhans giant cells were seen in the reaction of the tuberculous animal. Small numbers of lymphocytes were present in the reaction in both animals.

In the normal animal studied 14 days after injection of the tuberculo-phosphatide the reaction was almost wholly of epithelioid cells. These were of the second and third stages. There were a few Langhans giant cells, a few lymphocytes, and very occasional eosinophiles. In the larger reaction in the tuberculous animal the epithelioid cells were principally of the third stage; there were large numbers of giant cells, numerous neutrophiles, and small foci of caseous necrosis.

In summary of these results it may be stated that the reaction to the phosphatide was in general similar in the normal and tuberculous animals, that it was quantitatively greater, and qualitatively more

animals to the phosphatide 4 days after the injections showed the greater quantitative reaction in the tuberculous animal. In both there were numerous neutrophiles, though these were most prominent in the tuberculous animal. Monocytes and epithelioid cells of the first stage (with large vacuoles—sometimes identified as containing the lipid) were very numerous in both animals. As in the previous experiment, there was evidence of degenerative change in the carnosus, and fibroblastic proliferation adjacent thereto. In the normal animal there were clumps of epithelioid cells in the regional lymph node, and monocytes in the peripheral sinuses; but this condition was not seen in the tuberculous animal.

At 8 days the cellular reaction in the tuberculous animal was much larger than in the normal, showed more giant cells, more neutrophiles, and more necrosis. In both animals the predominating cells were second and third stage epithelioid cells (with medium sized and fine vacuoles). Again a moderate reaction of epithelioid cells was found in the regional node of the normal but not of the tuberculous animal.

Twelve days after the injections there remained a great deal of necrosis in the reaction of the tuberculous animal with degenerative changes in the carnosus muscle and in nerves running through the section, with infiltration of the latter by neutrophiles. There were also a few giant cells and small foci of hemorrhage. In the normal animal the reaction was much smaller, showed no hemorrhage, no necrosis, and very few neutrophiles. In both animals the epithelioid cells were predominantly of the third stage. Again epithelioid cells were found in the draining node of the normal animal. The corresponding node in the tuberculous animal showed extensive involvement with the disease, so that it was not possible to ascertain if there was reaction to the phosphatide in it.

After 18 days the reaction to phosphatide in the tuberculous animal showed no necrosis, but still a large number of third stage epithelioid cells, numerous giant cells, monocytes, fibroblasts, and a few eosinophiles. There was a reaction in the regional lymph node also but this may have been due to the disease. In the normal animal the only residuum was a few clumps of third stage epithelioid cells.

Twenty-two days after the injections both normal and tuberculous animals showed degenerative change in the carnosus with evidence

of regeneration and adjacent fibroblastic proliferation.   In the tuberculous animal there were numerous foreign body giant cells in such areas.   The only other residual reaction to the phosphatide in the normal animal was the presence of a few lymphocytes and moderate numbers of epithelioid cells.   The latter exhibited pyknotic nuclei and foamy cytoplasm.   But there were still large numbers of epithelioid cells in the reaction of the tuberculous animal, numerous Langhans giant cells, lymphocytes, and moderate numbers of eosinophiles.

Thus again the reaction to phosphatide in normal and tuberculous animals was qualitatively similar, but it differed in being more extensive in the tuberculous animal and characterized by more necrosis and by hemorrhage.   Also at the time the final observations were made, 22 days, the reaction in normal animals had largely regressed, while that in the tuberculous persisted.

The cellular reaction in the normal animal 24 hours after the injection of tuberculo-protein was moderate and was characterized by monocytes and neutrophiles in about equal numbers, with a few eosinophiles.   There were moderate microscopic edema and a little hemorrhage.   The reaction in the tuberculous animal was far more extensive, with a great deal of edema and hemorrhage, and greater numbers of neutrophiles.   Many of the latter were degenerating or dead, as determined by vacuolization and nuclear staining in supravital films or by nuclear changes in fixed sections.

At 48 hours, in the tuberculous animal the reaction to the tuberculoprotein showed marked edema with spreading apart of the fibers of the carnosus and considerable extravasation of red blood cells.   The cells in the extensive reaction were predominantly neutrophiles with smaller numbers of monocytes and eosinophiles.   The regional lymph node showed many monocytes, neutrophiles, eosinophiles, and a few myelocytes in the sinuses.   In contrast to this, the cellular reaction in the normal animal at this time was much less extensive and the monocytes predominated over the neutrophiles.   There was no hemorrhage and little evidence of edema.   Here also there were evidences of the reaction in the regional lymph node.

After 4 days the only reaction to be seen in the normal animal was an occasional small clump of monocytes.   But in the tuberculous animal the reaction was still quite extensive.   ·There was rather

extensive microscopic necrosis. The edema was less marked but the reaction of neutrophiles and monocytes persisted and there was an occasional cell resembling an epithelioid cell. There was evidence of degenerative change in the carnosus and a few foreign body giant cells, probably engaged in digestion of the degenerated muscle fibers.

In the normal animal injected with tuberculo-protein and sacrificed on the 5th day, the only reaction to be seen was an occasional small clump of monocytes and a few foreign body giant cells. In the tuberculous animal there was rather marked fibroblastic proliferation just beneath the carnosus and in the same zone rather numerous foreign body giant cells. Superficial to the carnosus were a few neutrophiles, lymphocytes, and phagocytic macrophages, with many monocytes. No epithelioid cells were seen. The regional lymph node of this animal showed many neutrophiles in the peripheral sinus.

The tuberculous animal sacrificed on the 6th day showed a similar but still more marked reaction. There were necrosis and hemorrhage, but there were many neutrophiles, monocytes, a few foreign body giant cells, and an occasional unequivocal epithelioid cell. The carnosus and cutaneous nerves showed degenerative change and neutrophilic infiltrations. The fact that the reaction was more pronounced than at 5 days is probably attributable to individual variation. The normal animal sacrificed on the 6th day showed no reaction to the protein.

These results showed that the tuberculo-protein produced a reaction, albeit microscopic, in the skin of a normal animal, characterized first by neutrophiles, later by monocytes; and the whole regressed in about 4 days. Evidence of toxic action on muscle and nerves of the skin was slight. But in tuberculous animals the reaction was greatly intensified and was accompanied by edema, hemorrhage, necrosis, and degenerative changes in muscle and nerve. Moreover, in the late reaction in the tuberculous animal there were numerous giant cells and occasional epithelioid cells. One could not be certain whether these had arisen *in situ* or migrated out from a tuberculous focus elsewhere in the body.

Intradermal Reactions to Tuberculo-Phosphatides Differing in Purity

Experiment 3.—Two preparations of tuberculo-phosphatide prepared for us by Dr. R. J. Anderson were used in this experiment. The first, designated A-3,

was prepared in the usual manner and purified by filtration of the ethereal solution of lipid through a Chamberland filter. The second preparation, designated A_3B was prepared from the first, "by dispersing in water, alcohol and ether; ... this solution was filtered through a new Mandler filter. Extraction with ether from the filtrate failed as the phosphatide was retained strongly in the colloidal aqueous solution. Magnesium sulphate, added to the solution, caused the phosphatide to coagulate. It was then extracted with ether, dried over Na_2SO_4, filtered, and concentrated to small volume, after which the solution was poured into cold acetone. The precipitate was filtered off, washed with acetone, and dried."[4] This preparation was a very fine white powder, whereas the A-3 was slightly cream-colored, granular, and quite hygroscopic. Each dispersed nicely to make an opalescent suspension. Films of each suspension, stained by the Ziehl-Neelsen method, showed only amorphous, non-acid-fast material and nothing having the appearance of bacteria.

These two preparations were suspended in sterile physiological saline so that 1 cc. contained 10 mg. Injections were made in each of six normal and six tuberculous guinea pigs, the dose of each injection being 1 mg. in 0.1 cc. Two normal and two tuberculous animals received the phosphatide A-3 intradermally on each side; two normal and two tuberculous received the phosphatide A_3B on each side; and two normal and two tuberculous received the phosphatide A-3 on one side and the A_3B on the opposite side. The areas of dermal reaction were measured 1, 4, and 9 days after the injections. One of each pair of animals was sacrificed for study 11 days following the injection, and the remainder 28 days after injection. This experiment was done to compare again the reactions in normal and tuberculous animals, to compare the reaction to the original phosphatide with that to the purified phosphatide, and to study the late cellular reaction to each.

As in prior experiments, the maximal cutaneous reaction in most of the animals occurred on the 4th day after injection. The reactions were much smaller than in earlier experiments, since the dose was much smaller. But again the reaction in tuberculous animals exceeded that in the normal. In both normal and tuberculous animals the reaction to the phosphatide A-3 was slightly greater than to the more highly purified A_3B. Also in all the tuberculous animals the maximal reaction was much smaller than would be obtained with the same dose, or even one-tenth this amount of the tuberculo-protein MA-100 used in the previous experiment. The maximal reaction observed even in tuberculous animals was 1.3 cm. in diameter. None of the reactions exhibited macroscopic necrosis.

Since half the animals in this experiment were sacrificed 11 days following injection of the phosphatide, and the remainder 28 days

[4] Personal communication from Dr. R. J. Anderson.

after the injections, much of the granulocytic reaction was not seen. In the tuberculous animals sacrificed at 11 days, a few neutrophiles, lymphocytes, and eosinophiles were to be seen in the reactions but the cells composing the greater part of the reaction were epithelioid cells and giant cells. Fig. 3 shows the reaction in a tuberculous animal sacrificed at this time and, together with Fig. 4, illustrates that the reaction was more complex than in normal animals (Fig. 1) in that there were more neutrophiles and eosinophiles. Fig. 2 shows the presence of the diffuse reaction in the dermal connective tissue, with edema and some degeneration of the connective tissue fibers. The compact area of reaction just superficial to the carnosus in Fig. 2 corresponds to an area such as is shown in Figs. 3 and 4. The reaction to the phosphatide A-3 was, in both normal and tuberculous animals, similar to the reaction to the phosphatide A_3B, but slightly greater. The reaction to both preparations in the normal animals was distinctly smaller than in the tuberculous animals, showed few or no neutrophiles and no necrosis, but was otherwise identical. Fig. 1 shows the reaction occurring in the skin of a normal guinea pig sacrificed at this time. By comparing with Figs. 2, 3, and 4, it may be seen that the reaction in normal animals was less intense and less complex cytologically.

In all of the animals injected intradermally with the phosphatide the reaction was found to be entirely superficial to the carnosus, and most compact and dense adjacent to it; but foci of reaction occurred throughout the dermis, being diffuse in the compact connective tissue, as shown in Fig. 2, but sometimes appearing as nests of cells in the more loose superficial connective tissue just beneath the epithelium. Fig. 5 illustrates the latter.

In the animals sacrificed 28 days after injection of phosphatide, the reactions to the two lipids were qualitatively similar but again quantitatively a little greater with the A-3. And again the reactions to each lipid were greater in the tuberculous than in the normal animals. All the reactions in both normal and tuberculous animals were diminished by about 90 per cent from the 11 day reaction. The necrosis and neutrophilic response were missing at this time, even in the diseased animals, so that the remnants of the reaction consisted solely of epithelioid cells and giant cells, both being the less numerous in normal animals.

Capacity of the Phosphatide to Induce Tuberculin Hypersensitiveness

We had found previously (1) that the phosphatide prepared by Dr. Anderson did not induce hypersensitiveness to tuberculin; but other workers (12, 13), using lipid extracts prepared in a similar manner, had found that these extracts induced hypersensitiveness to tuberculin. The following experiment was done to study again the possibility that the phosphatide of Anderson might induce hypersensitiveness to tuberculin.

Experiment 4.—Two normal guinea pigs were injected intraperitoneally with 8 mg. of human tubercle bacilli, strain H-37, after the organisms had been heated for 1 hour at 60°C. Two guinea pigs were injected intraperitoneally with 0.5 mg. of tuberculo-phosphatide, the amount which would be contained in the quantity of tubercle bacilli given to the two previous animals. Two other normal guinea pigs were given 4 mg. each of the phosphatide intraperitoneally, a quantity eight times as great as that in the tubercle bacilli given to the first two animals. These six animals were skin-tested intradermally with 0.1 mg. tuberculo-protein MA-100, 3, 6, 12, and 15 weeks after injection. A normal animal which had been injected intradermally with 2 mg. of Kieselguhr was similarly tested at the same time that the third and fourth tests were done. The tests were read according to the criteria set forth by Hetherington, McPhedran, Landis, and Opie (17), that is:

Any test showing edema at 48 hours is positive

Redness without edema......................................	negative
Edema less than 1 cm. in diameter............................	+
Edema 1 to 1.5 cm. in diameter...............................	++
Edema 1.5 cm. or greater without necrosis......................	+++
Extensive edema with spot of necrosis..........................	++++

The results of the tests are shown in Table IV.

The mild reactions on the third and fourth tests in the animals receiving the phosphatide were undoubtedly due to slight hypersensitiveness induced by the repeated test injections of tuberculo-protein. Especially is this probable in view of the positive reaction on the second test in the animal which received Kieselguhr. (The MA-100 protein is known to be capable of inducing hypersensitiveness (18, 19).) It seems more likely that this is the explanation than that sensitization due to the phosphatide finally became manifest after 12 weeks, and only then in the two animals which received the smallest dose of the lipid; but later in one other animal. However, hypersensitiveness was manifest in 3 weeks, with the *first* injection of tuber-

culo-protein, in the two animals receiving the amount of tubercle bacilli corresponding to the smaller dose of phosphatide.   It seemed probable, therefore, that the hypersensitiveness induced by tubercle bacilli was due to something not contained in the phosphatide, or present in such small quantity as to be incapable alone of inducing hypersensitiveness.   As a final check, however, the following was done.

Four normal guinea pigs were injected intradermally on the left side with 5 mg. of tuberculo-phosphatide in 0.12 cc. sterile saline.   The preparation of phosphatide

TABLE IV

Tuberculin Tests to Determine Capacity of Phosphatide to Induce Allergy to Tuberculin

Animal No.*	Injected with	Intradermal tuberculin test			
		3 weeks	6 weeks	12 weeks	15 weeks
R 5465	0.5 mg. phosphatide intraperi-	0	0	+	+
R 5466	toneally	0	0	+	++
R 5467	4.0 mg. phosphatide intraperi-	0	0	0	+
R 5468	toneally	0	0	0	0
R 5469	8 mg. killed bacilli intraperi-	+++	+++	+++	+++
R 5470	toneally	++	++	++	++
R 5471	2.0 mg. Kieselguhr intradermally	Not done	Not done	0	++

* These are serial numbers used in various experiments over many years.

used in this experiment contained a small amount of amorphous acid-fast material and a very few tubercle bacilli,—as near as could be determined, about thirteen bacilli per mg. of phosphatide.   Two of the guinea pigs were tested intradermally 3 weeks later, and the others 6 weeks later with 0.1 mg. of the MA-100 tuberculo-protein.   The tuberculin tests in these animals were all negative.   It is therefore apparent that, by the methods used, tuberculo-phosphatide preparations which contain no tubercle bacilli, or which contain only a very few, do not induce hypersensitiveness to tuberculin.   But preparations which are apparently bacteria-free do induce characteristic cellular responses in normal animals and accentuated reactions resembling the Koch phenomenon in tuberculous animals.

DISCUSSION

The cellular reaction to tuberculo-phosphatide injected intradermally or subcutaneously in normal animals is characterized macroscopically by the development during 3 or 4 days of a firm, slightly erythematous nodule which does not ulcerate and gradually regresses completely, leaving no scar. In tuberculous animals the nodule appears somewhat earlier, reaches its maximum size sooner, and is larger and more erythematous than in normal animals.

Microscopically the first response in normal animals is an outpouring of neutrophilic leucocytes, followed very quickly by infiltration and local proliferation of monocytes. The latter engulf the lipid and in so doing become epithelioid cells. Late in the reaction there are usually some lymphocytes and eosinophiles. After 2 weeks there is microscopically notable evidence of gradual regression of the reaction, the epithelioid cells showing pyknosis and vacuolization of the periphery of their cytoplasm. With small doses regression is almost complete in 1 month. In tuberculous animals there is much greater exudation of granulocytes, there are some edema, slight hemorrhage, necrosis, and degenerative changes in muscle, nerve sheaths, and connective tissue. But the cytologic evolution of the reaction is similar to that in normal animals, albeit accelerated and characterized throughout by numerous neutrophiles. The epithelioid cells dominate the reaction after about 4 days, and persist longer than in normal animals so that there remains a considerable reaction at the end of 1 month. Thus, in the tuberculous animal the accelerated augmented response is similar in many respects to the Koch phenomenon. The greater response in tuberculous animals is very likely due to a modification of the connective tissues of the entire body; it may be non-specific since Geiger (20) has noted augmented hematologic responses to non-specific agents.

Single injections of 0.5 mg. to 5 mg. of tuberculo-phosphatide introduced either intraperitoneally or intradermally fail to induce hypersensitiveness to tuberculin. Other experiments reported by Sabin and Joyner (21) show that hypersensitiveness to tuberculin is not induced by repeated intradermal injections of the lipid, the total amount being 30 mg. given in six doses of 5 mg. each.

By contrast with the reaction to the phosphatide, the reaction to tuberculo-protein (water-soluble MA-100) in normal animals is not visible macroscopically. Microscopically the response is moderate, characterized by neutrophiles and monocytes in about equal numbers at 24 hours, and thereafter by relatively and actually fewer neutrophiles. The monocytes are not stimulated and do not become epithelioid cells. The reaction regresses rapidly and leaves no visible residue in 6 days.

In tuberculous animals the response to tuberculo-protein is marked and rapid. It may be progressive for as long as 4 days, after which there is gradual regression. Microscopically there are marked edema at 24 hours, small foci of hemorrhage, and intense cellular reaction of neutrophiles and smaller numbers of monocytes. Many of the neutrophiles show degenerative change. This reaction persists and by the 4th day there is evidence of degenerative change also in connective tissue and muscle. Also after 4 or 5 days there may be a few epithelioid cells, but they are not massive in number as in the reaction to the phosphatide. There is fibroblastic proliferation, especially adjacent to injured muscle, and a few foreign body giant cells may be seen. The neutrophilic leucocytes persist throughout the reaction and dominate the cellular picture.

Some of these results differ from those of other workers (12, 13), and our interpretations are also at variance with some others (16). The capacity of phospholipids prepared by others to induce hypersensitiveness to tuberculin is undoubtedly due to the presence of tubercle bacilli in considerable quantity in these preparations. The preparations used in our cellular studies were apparently bacteria-free, yet yielded characteristic cellular responses. The difference in results (12, 13) is in all probability due to differences in methods of preparing the lipid fractions.

The interpretation made by Holley (16) that the reaction to phosphatide is due to protein accompanying or bound to the lipid seems untenable, since the reaction to the protein in normal animals is not characterized at all by epithelioid cells, whereas the same dose of phosphatide induces a larger reaction characterized chiefly by epithelioid cells. Moreover, the slight reaction of epithelioid cells and foreign body giant cells occurring late in the response of tuberculous

animals may be an indirect response, *i.e.*, a reaction to degenerated cells, and necrotic tissue, since in the normal animals, in the absence of necrosis and degeneration, these are not seen.

SUMMARY

Prior observations on the cellular reactions to tuberculo-phosphatide are confirmed and compared with reactions induced by this material in tuberculous animals. In the latter the response is accelerated and augmented and simulates the Koch phenomenon.

Tuberculo-protein produces no macroscopic reaction in normal animals. The microscopic reaction of neutrophiles and monocytes regresses in less than a week. The same material in tuberculous animals causes a response characterized by more or less hemorrhage and necrosis, tissue degeneration, and infiltration of neutrophiles and monocytes. Late in the reaction there may be a few epithelioid cells and foreign body giant cells.

Preparations of tuberculo-phosphatide which contain no tubercle bacilli, or only a few, induce the typical cellular response but do not induce hypersensitiveness to tuberculin.

Repeated intradermal skin-test injections of tuberculo-protein MA-100 in normal guinea pigs may be followed by a mild hypersensitiveness to subsequent injections.

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EXPLANATION OF PLATE 28

Fig. 1. Section of skin from a normal guinea pig (R 5475), sacrificed 11 days following the intradermal injection of 1 mg. of purified tuberculo-phosphatide A_3B. The reaction consists almost wholly of epitheloid cells and monocytes lying superficial to the carnosus, in the dermis. Dr. N. C. Foot's modification of Masson trichrome stain. $\times$ 150.

Fig. 2. Section of skin from a tuberculous guinea pig (R 5391), sacrificed 11 days following the intradermal injection of 1 mg. of tuberculo-phosphatide A-3. Diffuse cellular reaction in cutaneous connective tissue, where there are also degenerative changes in the fibers, and edema. Intense cellular reaction of epithelioid cells, monocytes, neutrophiles, and eosinophiles just superficial to the carnosus. Stain same as above. $\times$ 50.

Fig. 3. Section of skin from a tuberculous guinea pig (R 5389), sacrificed 11 days following intradermal injection of 1 mg. of the purified tuberculo-phosphatide A_3B. Photograph shows mixed character of the reaction, with giant cells, epithelioid cells, and neutrophiles, the latter being more numerous than in normal animals. The area enclosed by the squared lines is enlarged in Fig. 4. Stain same as above. $\times$ 150.

Fig. 4. Area indicated by squared lines in Fig. 3 at higher magnification, to show a focus of neutrophiles and monocytes in the reaction. $\times$ 700.

Fig. 5. Section of skin from tuberculous guinea pig (R 5393), sacrificed 11 days after the intradermal injection of 1 mg. of the purified tuberculo-phosphatide A_3B. Photograph shows reaction of epithelioid cells lying in the loose dermal connective tissue, immediately under the cutaneous epithelium. Stain same as above. $\times$ 200.
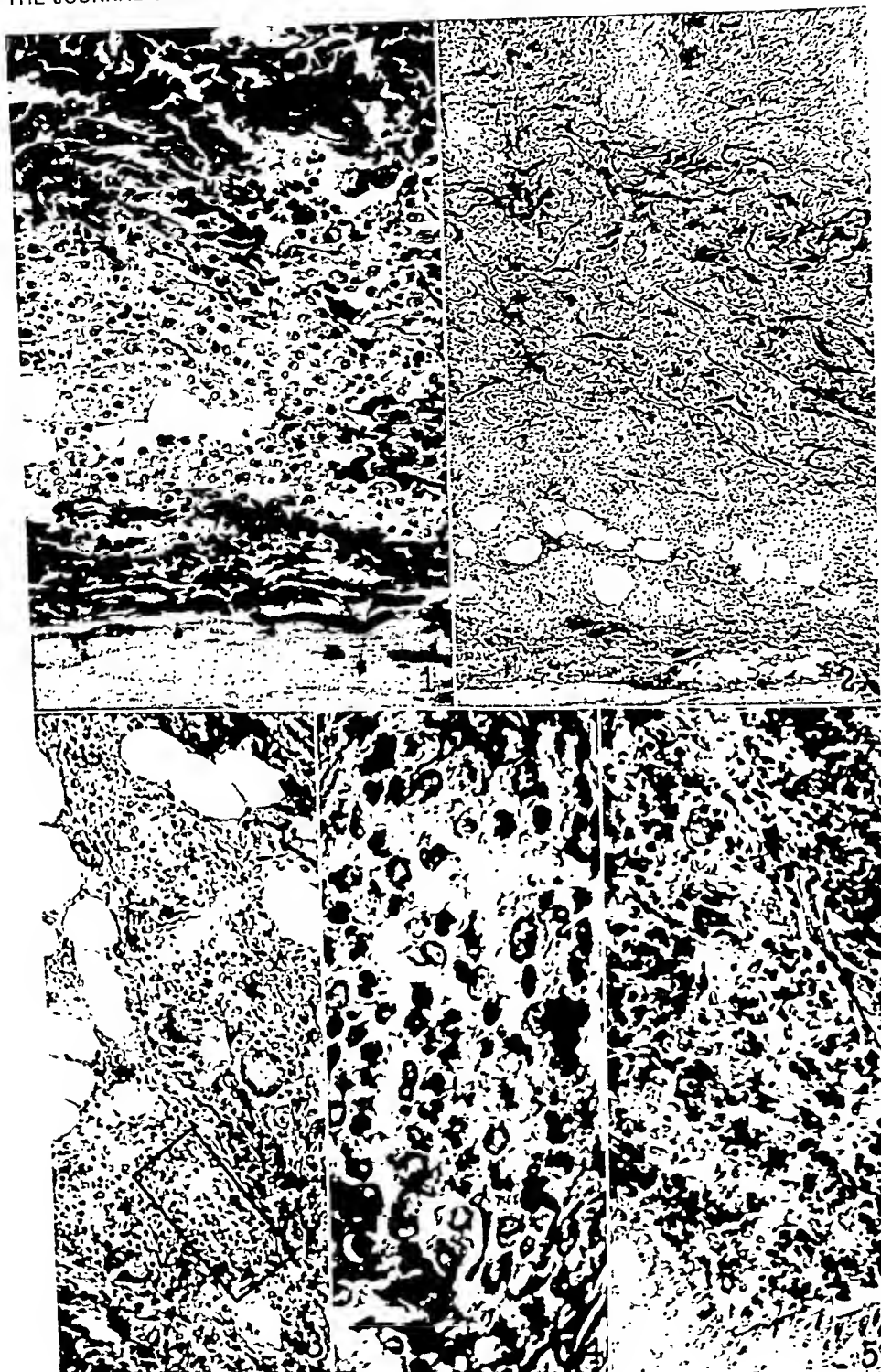
(Smithburn and Sabin: Tuberculo-protein and tuberculo-phosphatide

TUBERCULAR ALLERGY WITHOUT INFECTION*

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For a period of years we have been studying the effects of materials from tubercle bacilli on the tissues of normal and tuberculous rabbits and guinea pigs. The present study is the beginning of the project to use some of the fractions together to see if the presence of two substances in the tissues modifies the effect of either given alone. In the experiments described here it has been found that the addition of tuberculo-phosphatide to tuberculo-protein enhances the sensitization to the protein to a marked extent, producing, after three or four injections of relatively small amounts of protein, a degree of sensitization like that of the disease.

The subject of sensitization in connection with tuberculosis has a long and complex history.

In 1910 and 1911 Baldwin (1) published important studies on the nature of tuberculin sensitivity in which he concluded that the presence of tubercles was necessary in order that tuberculo-protein should elicit skin reactions like those of the disease. He found that after repeated intraperitoneal injections of tuberculo-protein (filtered water-extract) into both rabbits and guinea pigs they exhibited a form of anaphylactic response, that is, they would die if the same extract were injected intravenously or intracerebrally but they did not react locally to intradermal injections. He used accessory materials to induce the skin-sensitizing

power of the protein, namely, charcoal, lipids extracted from tubercle bacilli with benzol, and beeswax, but with negative results. On the other hand, he noted that when tubercles were formed, even by killed tubercle bacilli, sensitization occurred. Hence he concluded that " . . . if tuberculin reactivity is present in any animal, the presence of tubercles must be predicated" (1 b). This concept was confirmed and strengthened by Krause (2).

It is now firmly established that skin sensitivity can be induced by heat-killed tubercle bacilli, through the early work of Borrel (3), of Baldwin (1 b) just referred to, and of Bessau (4), and extending to the more detailed and conclusive studies of Petroff (5), Petroff and Stewart (6), and Lange and Freund (7).

Many failures to sensitize animals so that they would give a local reaction to an intradermal injection with tuberculo-protein have been reported, but since Dr. Seibert and Drs. Heidelberger and Menzel have now extracted more highly antigenic tuberculo-proteins the negative evidence need not be reviewed. However, two points have been brought out in this recent work that may help to explain the discrepancies with previous reports. First, both Seibert (8 a) and Heidelberger and Menzel (9) have shown that differences in methods of preparation of these proteins give modifications in the products. Second, Seibert (8 b–c) has shown that the heating used in the preparation of the so called old tuberculin (OT) changes the size of the active protein molecule, the molecular weight of which is about 32,000, to a molecule the weight of which is about 16,000 and which is not an effective antigen by itself, and yet is able to elicit a skin reaction in the already sensitized animal.[1]

The fact that an accessory substance may enhance the antigenicity of a protein has been reported recently. Some of the materials used have been relatively inert, such as carbon particles or alum to which the protein is probably adsorbed; another group has been lipids which may give rise to more complex cellular changes. In 1926 Gaehtgens (10) reported that the use of a mixture of serum and lipid (alcoholic extracts of tissues) injected into rabbits induced antisera with a higher precipitin titer than the serum alone. This was not confirmed by Dresel and Meissner (11) who reported their observations in 1927. Tytler (12) summed up previous studies on the antigenic power of tuberculo-protein as inconclusive but stated that he could elicit sensitization by incorporating dry bacillary tuberculo-protein in beeswax and injecting them together. In this way he obtained excellent skin sensitization with necrosis and noted that animals with the stronger reactions showed a flare-up of previous reactions on reinjection with the wax-protein.

In 1935 Saenz (13 a) showed that dead tubercle bacilli enveloped with petro-

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[1] These are the figures from Dr. Seibert's recent studies with the ultracentrifuge technique in Professor T. Svedberg's laboratory at Upsala. Her results have recently been published (Seibert, F. B., Pedersen, K. O., and Tiselius, A., *J. Exp. Med.*, 1938, 68, 413).

leum jelly induced allergy sooner, that is, in from 6 to 8 days, and of more intense degree than dead bacilli in saline, or living bacilli. He interpreted this to mean that the oil induces an inflammatory reaction that favors the increase in allergy. In 1937 he showed (13 b) that with small doses of bacilli in the presence of oil there was a slight reduction in the dispersion of the bacilli.

In 1935 Seibert (8 d, e) showed that a mixture of the purified protein derivative (that is the preparation of the smaller molecules) with aluminum hydroxide or carbon particles produced a complete antigen. She interpreted this result to mean that the antigen is then composed of particles of aluminum hydroxide with adsorbed protein. The cellular reaction to aluminum hydroxide (Willstätter type C), inducing epithelioid cells forming tubercle-like structures, has been followed by Olitsky and Harford (14) and by Jørgensen (15). Jørgensen discusses the immunological significance of this type of cell reaction. The foreign body reaction to carbon particles is well known.

Materials and Methods

We obtained from Dr. Seibert three preparations of tuberculo-protein designated TPA, SOTT, and PPD. The TPA is a purified tuberculo-protein precipitated by ammonium sulfate and separated in large part from free polysaccharide (Seibert, 8 a, d, e; and Seibert and Munday, 16). It is the equivalent in potency and purity of Seibert's newer preparation of protein designated TPT which is prepared by precipitation with trichloracetic acid. The SOTT and PPD are also equivalent preparations as regards tuberculin activity. SOTT (the letters signifying synthetic-media; old-tuberculin; trichloracetic-acid-precipitate) was the original designation for the material now called purified protein derivative, PPD. This preparation is made from an old tuberculin obtained from synthetic media after growth of standard strains of human tubercle bacilli for from 6 to 8 weeks and heated for 3 hours in an Arnold sterilizer. The heating reduces the size of the protein molecule. The material is then submitted to ultrafiltration to remove salts, glycerine, etc., and finally is precipitated with trichloracetic acid to remove polysaccharide (Seibert, 8 c).

The proteins designated D and G were given to us by Drs. Heidelberger and Menzel (9). They were extracted from human tubercle bacilli, strain H-37. The D fraction was obtained with M/15 phosphate buffer at pH 6.5 after the frozen and dried bacilli had been extracted with acetone and with ether to remove the lipid and with buffer at pH 4 to acidify the proteins and remove free polysaccharide. The D fraction was then precipitated with acetic acid. The G fraction was obtained with water made alkaline to about pH 11.0 after previous extractions with less alkaline buffers. These processes were all carried on in the refrigerator to minimize enzyme action. These preparations are very low in free polysaccharide but contain bound polysaccharide because they elicit the formation of antibodies to the carbohydrate in rabbits, as well as antibodies to the protein.

The protein MA-100 was given to us by Dr. Reichel; it was prepared at the Mulford Laboratories of the Sharp and Dohme Company from synthetic culture media on which human tubercle bacilli, strain H-37, had been grown (Masucci and McAlpine, 17). This protein was obtained by precipitation with ammonium sulfate, as was Seibert's protein M-9 (Seibert and Munday, 16 a; and Seibert, 8 b).

The tuberculo-phosphatide and the yeast-lecithin used in these experiments were prepared by Dr. R. J. Anderson. The phosphatide was extracted from human tubercle bacilli, strain H-37, in 1932 (18). It was filtered through Chamberland candles; it is predominantly crystalline and has a nitrogen content of 0.4 per cent due to ammonia nitrogen. The methods by which we determined that the enhancement of sensitization was not caused by contaminating tubercle bacilli but by the phosphatide itself are discussed in this paper. The phosphatide gives rise to uniform and stable suspensions when rubbed in water. A suspension of the phosphatide in saline was made and added to the protein in solution just before making the injections. Aluminum hydroxide in the form of Willstätter's gel was furnished us by Dr. Peter K. Olitsky. This material, in the dilution used, contained combined aluminum in the amount of 15 mg. dry weight per cc.

All control materials, which were to be free of tuberculo-protein, were handled in new glassware which had never been in contact with tubercle bacilli nor any of their products. All dilutions of the tuberculo-protein were made just prior to performing the injections in order to insure no loss of potency.

We used guinea pigs in the experiments, whose average weight was about 300 gm. and they were grouped according to weight so that the average weight of each group corresponded to that of the other groups. At the suggestion of Dr. Karl Landsteiner we have used the intradermal route for the sensitizing injections and have limited the injections to the dorsal region where the skin is thickest. The injections were placed about 2 cm. apart and each succeeding one was made on the side opposite to the previous injection. Reactions to the injected materials and the final skin tests were measured with calipers at 24 hours and 48 hours.

RESULTS

These experiments were planned on the basis of two previous studies in sensitization, one by Dr. Seibert (8 a) and the other by Smithburn, Sabin, and Geiger (19). In 1932 Seibert reported that local cutaneous sensitization (Arthus phenomenon) could be induced in rabbits and guinea pigs with tuberculo-protein. Seibert used repeated intraperitoneal injections of 10 mg. of the protein. The least amount of the protein required to sensitize any rabbit was 47 mg. and other rabbits took as much as 225 mg. The least amount of protein required to sensitize any of the guinea pigs was 30 mg. In 1934 Smith-

burn, Sabin, and Geiger (19) reported sensitizing both rabbits and guinea pigs by repeated subcutaneous injections of tuberculo-protein (MA-100) over a period of 13 weeks. The total amount of protein used in the rabbits was approximately 27 mg. and in guinea pigs, 5 mg.

In the present experiments we used phosphatide in the hope of reducing the amount of tuberculo-protein necessary to sensitize in order to eliminate the discrepancies between artificial sensitization and that occurring in the disease.

These studies are presented through three experiments, first, a comparison of the reactions of guinea pigs to intradermal injections of the protein MA-100 by itself and as enhanced with tuberculo-phosphatide, lecithin, and aluminum hydroxide. Earlier experiments in sensitization in this laboratory had been made with MA-100 (Smithburn, Sabin, and Geiger, 19). Second, a study of the two more highly antigenic tuberculo-proteins, the TPA of Seibert and the D fraction of Heidelberger and Menzel, used with and without the tuberculophosphatide; and third, the effect of the phosphatide mixed with the protein derivatives SOTT and PPD of Seibert.

A general survey of the power of the tuberculo-phosphatide to enhance sensitization is shown on Chart 1.

For this group of animals the quantity of phosphatide was 5 mg. and the protein 0.5 mg. in 0.1 cc. saline for each injection and there were six injections. For the final test of sensitization 0.1 mg. of MA-100 (the standard test dose for guinea pigs) was used for all the animals. These tests were made a week after the last sensitizing injection. The standards for estimating the skin tests were those generally accepted as stated by Hetherington, McPhedran, Landis, and Opie (20) for human reactions. The standard is that a reaction called one plus is an area of edema measuring 10 mm. in diameter; a two plus reaction has more marked edema and measures from 10 to 15 mm.; the three and four plus reactions both have more marked edema and measure more than 15 mm., but the three plus reaction lacks, while the four plus reaction is characterized by necrosis.

The basic control for the use of this phosphatide to enhance sensitization to tuberculo-protein consisted in determining that this phosphatide by itself did not sensitize to the protein. Six guinea pigs received six dorsal intradermal injections of 5 mg. of phosphatide at intervals of 1 week. The mean size of these reactions 24 hours after the injections did not vary appreciably, as is illustrated in the second line of Chart 1, and all six were negative to the final test with the protein MA-100.

The phosphatide was obtained by Dr. Anderson from the bacilli by means of

appropriate solvents, but, though by these means he could separate this lipid from other lipids, such as fatty acids and waxes (alcohols), a few acid-fast bacilli remained in the first preparation of the phosphatide. To remove these residual bacilli, he filtered the material, in a lipoidal solvent, through Chamberland candles.

Mean size of reactions

No. of Guinea pigs	Materials used to sensitize	1 2 3 4 5 6 cm.	Reaction to 0.1 mg. MA-100
5	MA-100		3+ to 1+
6	Phosphatide		Neg.
5	Phosphatide + MA-100		3+
6	Phosphatide + PPD		3+ to neg.
6	Phosphatide + S0TT		3+ to 1+
3	TPA		3+ to 2+
3	Phosphatide + TPA		3+
3	Phosphatide + D		3+ to neg.

Weeks
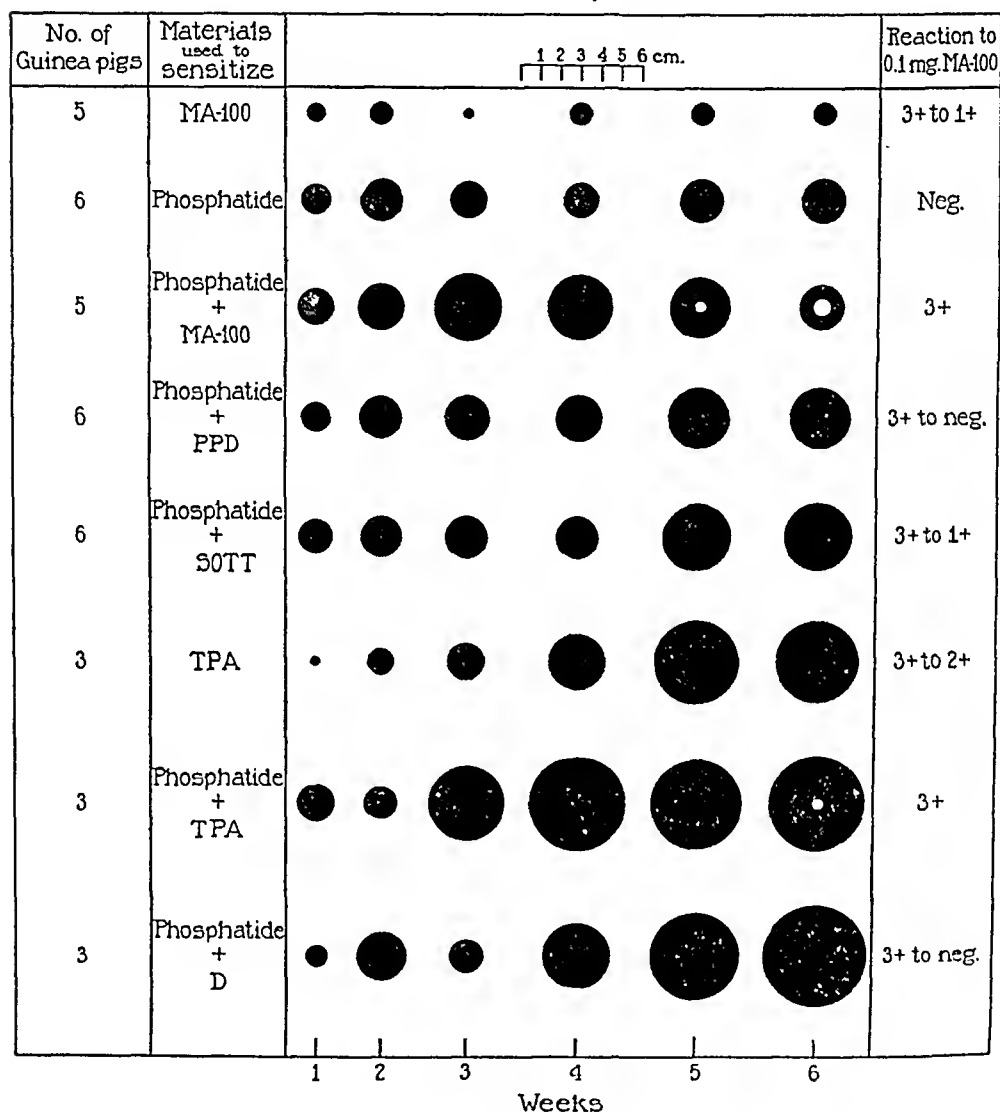
CHART 1

The use of a lipoidal solvent in this procedure alters the conditions of filtration to such an extent that all of the bacilli are not prevented from passing through the candles in every filtration. Repeated attempts to demonstrate acid-fast bacilli in stained preparations of the phosphatide used in these experiments have been

negative. Such negative results, however, cannot be considered as establishing the complete absence of bacilli in the phosphatide. As a further check we dissolved 200 mg. of this phosphatide in a mixture of three parts of chloroform and seven parts of ether, proportions that give a fluid, the specific gravity of which allows sedimentation of tubercle bacilli. This solution was then centrifuged at high speed for 4 hours and in the sediment we were again unable to find acid-fast bacilli. As a further check we devised the following experiment. The amount of protein containing nitrogen in an amount equivalent to the total nitrogen content per milligram of phosphatide was computed and added to the phosphatide. Eight guinea pigs then received ten daily intradermal injections of this mixture, 0.1 mg. of phosphatide with 0.0025 mg. of protein, making a total of 1 mg. of the phosphatide and 0.025 mg. of the protein. 25 days after the last injection, all of the animals were tested with 0.1 mg. MA-100, with the following results. In this experiment four of the guinea pigs received the protein, which was the Heidelberger-Menzel type G in the form of an alum precipitate. When tested with MA-100, one of these was negative and three showed three plus reactions (measuring 741, 966, and 1050 sq. mm.); the other four animals received the same protein in solution and the tuberculin test showed three to be negative while one showed a reaction which measured 156 sq. mm. and was considered two plus. On the basis of these tests, with four out of eight guinea pigs sensitized with the addition of so small an amount of protein, namely, 0.025 mg., we consider it legitimate to have used this preparation of phosphatide as an enhancing agent comparable to the other materials, lecithin and aluminum hydroxide.

Experiment 1.—The mean size of the reactions 24 hours after intradermal injections of protein MA-100 is given on the first line of Chart 1 and the reactions to the same protein plus phosphatide on the third line. Evidences of sensitization, that is, of changed reactions, are indicated in three ways on the chart: first, by a change in the size of reaction to the successive weekly injections of the same antigen; second, by the appearance of necrosis which is signified by the white circles in the center of the black zones; and third, by the effect of the final test injection with 0.1 mg. of protein MA-100, recorded in the last column.

With the MA-100 there was relatively little change in the mean size of the reactions on repeated injections, showing that this preparation is a less potent antigen than protein TPA. This observation confirms the results obtained by Seibert (8 b). This is also borne out by the fact that in the final test with the same protein, only one of the five guinea pigs exhibited a three plus reaction, while two of them were two plus and two were one plus. This protein, however, when used with the phosphatide, was a good antigen, showing an increase in the size of the reaction after the second injection and giving well marked necrosis after the fifth and sixth injections. After the six injections of MA-100 plus phosphatide, all of the guinea pigs gave three or four plus reactions on testing with the MA-100 alone. These data are clear evidence of the power of the phosphatide to enhance the sensitizing potency of protein MA-100.

On Table I is summarized the comparison of the power of two other materials

not obtained from tubercle bacilli, namely, lecithin from yeast and aluminum hydroxide, with the power of tuberculo-phosphatide to enhance the sensitizing power of MA-100. None of the three accessory materials given alone induced any significant increase in the size of the reaction to the repeated injections and none of them sensitized to tuberculo-protein, but all three enhanced the sensitizing power of the protein, lecithin, and aluminum hydroxide to a moderate degree and tuberculo-phosphatide to a striking degree. Both lecithin (Sabin, Doan, and Forkner, 21) and aluminum hydroxide (Olitsky and Harford, 14 a and b) induce the formation of epithelioid cells.

TABLE I

Comparison of Lecithin and Aluminum Hydroxide with Tuberculo-Phosphatide in Power to Enhance Sensitization to Tuberculo-Protein MA-100

Number of animals	Material	Mean size of reactions						Reaction to 0.1 mg. of MA-100
		1st injection	2nd injection	3rd injection	4th injection	5th injection	6th injection	
		sq.mm.	sq.mm.	sq.mm.	sq.mm.	sq.mm.	sq.mm.	
5	MA-100	87	106	21	124	120	100	3+ to 1+
6	Phosphatide	317	412	329	289	432	460	Negative
5	Phosphatide and MA-100	344	482	1114	1064	913	584	3+
6	Lecithin	150	178	169	226	240	237	Negative
5	Lecithin and MA-100	270	414	599	481	283	530	3+ to negative
6	Aluminum hydroxide	89	74	101	115	109	116	Negative
6	Aluminum hydroxide and MA-100	204	310	460	513	1000	752	3+ to 2+

Experiment 2.—We have also used two highly antigenic proteins in these experiments, Seibert's TPA and the D fraction of Heidelberger and Menzel, as shown on Chart 1. The reactions to the protein TPA steadily increase in size with each weekly injection, indicating that the material is a potent antigen. With the addition of phosphatide, however, there was a more rapid increase in the size of the local reactions. This was but a small part of the difference in reaction to the reinforced protein, for there were qualitative differences of still greater significance. With the protein by itself reactions were elicited which did not go on to necrosis, after the number of injections used in these experiments, and they were faded in 48 hours; while, after the mixed injections, the reactions showed marked necrosis in 24 hours and persisted for the 48 hour period.

These differences were apparent both on inspection of the lesions in the animals and on study of sections of the material. A histological study of the reactions was made in a second series of guinea pigs which received successive injections of the protein and the protein plus phosphatide in the same dosages as previously and at the same intervals. The local reactions in the skin 24 hours after the injections of the protein are shown on Figs. 1 to 4 and the corresponding series for the mixed injections on Figs. 5 to 8. In this series one animal of each group was sacrificed 24 hours after each sensitizing injection for histological studies. Other guinea pigs received only one injection and were sacrificed at varying intervals for study of the residual reaction.

As usual, the first injection of the protein alone gave a negative reaction, as determined by gross inspection; its site, as seen in the animal (indicated by the arrow on Fig. 1) was scarcely more than a needle puncture. Quite different, on the other hand, was the response of the mixture (Fig. 5). In sections the sites of the two injections also showed a marked contrast, as can be seen in Figs. 9 to 11. In Fig. 10 is shown at low magnification the amount of the 24 hour reaction to a single injection of the protein plus phosphatide. In Fig. 9 is shown the densest zone of reaction to the protein alone, in the deepest layers of the dermis and the subcutaneous level, to be compared with a corresponding zone after the mixed injection shown in Fig. 11. After the protein alone there was a moderate infiltration of the tissues with neutrophilic leucocytes, most numerous just beneath the epithelium, and some increase in monocytes, especially in the subcutaneous tissues. As shown in the photograph, there was a perineural and perivascular infiltration with monocytes. After injection of the protein plus phosphatide the same types of cells were involved, but there was a much greater outpouring of neutrophiles and a much greater increase in monocytes (Fig. 10). Moreover, the monocytes had been altered functionally because they had phagocytized the phosphatide. This was shown in sections by a vacuolization of these cells, the lipid having been dissolved out of them in the processes of embedding. Previous studies in this laboratory (Sabin, Doan, and Forkner, 21; and Smithburn and Sabin, 22 a) have shown that phagocytosis of phosphatide can be the first step in the formation of epithelioid cells from monocytes. It is this very marked difference in cellular response to the phosphatide plus protein, as compared with the response to the protein by itself, that initiates the speeding up of sensitization to be made out with succeeding injections.

The reaction to a single injection of these two materials was studied in sections after an interval of 9 days. An effect of the protein alone was still demonstrable and readily discriminated, even with low power of the microscope, from the effect of the mixed injection. In both all traces of the neutrophilic lecucocytes had disappeared; the differences were in the number and the qualitative changes in monocytes. After the protein TPA alone there was some increase in monocytes over the number in normal skin and there were a few epithelioid cells. After the less potent protein MA-100 Smithburn and Sabin (22 b) did not find

any epithelioid cells. After the protein plus phosphatide there was a much greater residual reaction of monocytes and epithelioid cells. In both series there was an infiltration of the tissues with eosinophiles.

Still more striking differences are to be made out in the tissues after repeated injections of protein and protein plus phosphatide, that is, as the animals become sensitized. In Figs. 12 to 14 are illustrated the residual effect of a first injection of protein (Fig. 12) and of protein plus phosphatide (Figs. 13 and 14) taken 24 hours after a second injection had been made. Thus these reactions are 1 week old. 24 hours after the second injection of the mixture there was a lighting up of the first injection, the site of which could not be noticed in the animal at the time the second injection was made (Fig. 6). This flare-up of the site of the first injection was due to edema and hemorrhage. This phenomenon indicates already a difference in the sensitivity induced by the presence of the phosphatide.

The residual reaction to the protein alone, after 1 week (Fig. 12), shows a considerable increase in monocytes throughout the dermis over the number in the normal skin. They occurred in small foci comparable to the milk spots of the omentum, indicating a local increase in these cells rather than an infiltration from the blood stream. Even with the protein alone this residual injection must be considered in the light of sensitization inasmuch as the second reaction to an injection of the protein was larger than the first (Figs. 1 and 2).

The corresponding residual reaction of 1 week's duration to protein plus phosphatide, taken 24 hours after the second intradermal injection, shows both the direct effects of the materials injected and the effects of sensitization. The dermis and the subcutaneous level were dense with cells (Fig. 13), largely epithelioid cells in small foci or tubercles. Such a tubercle-like mass is to be seen below the center of Fig. 13; at higher magnification its center shows a fresh hemorrhage. The edge of this tubercle is shown in Fig. 14. In the sections of this material there were a few neutrophiles, some monocytes, and a considerable infiltration with eosinophiles, especially just under the epithelium. The epithelium was much thickened, as can be seen by comparing Figs. 10 and 13. This thickening of the epithelium was still more marked in some of the later injections, with an increase in the number of mitotic figures.

The site of the 24 hour reaction to the second injection of phosphatide plus protein was markedly different histologically from the corresponding 24 hour reaction to the first injection. The most striking change was in the presence of edema; the fibers were spread apart and it could be seen that they were swollen and in places had a changed reaction to the stain. The cellular infiltrations, both of neutrophiles and of monocytes, were much greater. It will be noted in Fig. 6 that there was a slight ulcer in the center of the reaction; this was probably due to a bleb of the injected material which had raised and damaged the epithelium. Beneath this ulcer there was a massive infiltration of the tissues with neutrophiles.

The reactions to the third injection were the same in kind but showed increas-

ing discrepancies between the simple and mixed injections. After the protein plus phosphatide there was an increasing formation of monocytes, extending not only throughout the dermis and subcutaneous levels but also beneath the carnosus muscle. Such a mass of young monocytes, with the area infiltrated with eosinophiles, is shown at this level in Fig. 15, 24 hours after a third injection. In this material there was considerable perivascular infiltration. The reactions to the third injections did not differ greatly in size, but the qualitative differences were marked. The lesion after protein alone (Fig. 3) was soft and flat; it was an erythema without much edema, whereas the site of the third injection of the mixture (Fig. 7) was markedly raised and was indurated. The thickness of the lesion is just suggested in the photograph by the relief given by a band of hemorrhage on the left border.

After the fourth injection of protein plus phosphatide, massive necrosis became the predominant difference between the two types of injections. This will be plain if Figs. 4 and 8 are compared. It will be noted that the site of the fourth, necrotic reaction to the mixed injection was decidedly smaller than that of the third injection. This has been a constant phenomenon, that is, as soon as the degree of sensitization has given marked necrosis, there has always been a reduction in the area of the lesion. Three out of four of the guinea pigs of this series receiving the mixed injection showed marked macroscopic necrosis after the fourth injection. In the series shown on Chart 1, necrosis became as marked only after the fifth injection. With massive necrosis the lesions became very complex. There was the increase in cells due to the materials injected; but besides these direct effects there were edema and hemorrhage, clotting of material in lymphatics, and swelling of the endothelium of the veins. The death of cells and the damage to fibers caused new infiltrations with neutrophiles and phagocytosis of debris. Ultimately there was replacement of the necrotic tissue with fibroblasts. These residual reactions were studied after intervals of 14, 22, 27, and 35 days. In 35 days there was little or no cellular reaction to be seen. When necrosis had been present the residual reaction was predominantly of scar tissue.

With a testing injection of 0.1 mg. of MA-100 we did not obtain necrosis, even in the case of animals which had shown necrosis after later injections of the phosphatide plus protein. Thus, as is shown on Chart 1, the groups of guinea pigs which received either MA-100 or TPA with the phosphatide showed only three plus reactions in the final test with MA-100. In another series of guinea pigs, however, the final test was made with 0.5 mg. of MA-100 and in these tests the highly sensitized animals did show four plus reactions, that is, with necrosis.

Dr. Seibert[2] has found that guinea pigs sensitized with tuberculoprotein TPA until they gave three and four plus reactions with the same antigen did not react to the purified protein derivative, PPD.

[2] Personal communication.

This is in agreement with the results of Boquet, Sandor, and Schaefer (23) who sensitized guinea pigs to tuberculo-protein and failed to elicit skin reactions in them with tuberculin. We have confirmed these results. A series of ten guinea pigs were sensitized with five injections of phosphatide (5 mg.) plus tuberculo-protein TPA (0.5 mg. in 0.1 cc. saline) intradermally every 4 days. To the fifth injection all of them showed either three or four plus reactions. The five which were the most highly sensitized were then tested with 0.1 mg. of PPD in saline and all of them were negative. The other five received another injection of the phosphatide plus protein and then all were inoculated subcutaneously with 0.1 mg. of virulent tubercle bacilli, human strain H-37. The entire group failed to show the Koch phenomenon. The animals were watched for $3\frac{1}{2}$ weeks, during which time they reacted like the normal controls; there was no immediate irritation to the injection of the bacilli; the nodules formed and ulcerated as did those of the non-sensitized animals.

DISCUSSION

For the thesis that tuberculo-phosphatide has the power to enhance sensitization to tuberculo-protein, it was necessary to establish the point that this power is due to the phosphatide itself and not to contaminating dead tubercle bacilli containing protein. If bacilli were present in adequate numbers they would certainly be a factor in inducing particular cellular reactions as well as sensitization. In some preparations of phosphatide other than the one used in these experiments, tubercle bacilli have been present and have been readily demonstrated both in films of the phosphatide and in giant cells induced in animals after injection of the material. However, in the preparation of phosphatide used in these experiments no bacilli were found. More conclusive than this negative evidence is the fact that a total of 30 mg. of this phosphatide alone, given in 6 weekly intradermal injections, failed to sensitize any of six guinea pigs to the protein, and the addition of 0.025 mg. of a tuberculo-protein to 1 mg. of the phosphatide produced a mixture that did sensitize four out of eight guinea pigs. This indicates that the preparation of phosphatide used in these experiments did not carry enough protein to be biologically significant and this makes valid the claim that the material

itself has the power to enhance the potency of the protein to sensitize.

It is clear that the active tuberculo-protein sensitizes guinea pigs when given by the intradermal route, as shown in the increasing reactions exhibited on Chart 1. The enhancement of this sensitization by the addition of tuberculo-phosphatide is shown by the greater size of corresponding reactions, by the lighting up of the site of previous injections, by the development of marked induration in the lesions, the early appearance of necrosis, and the fact that the reaction does not fade as quickly as when protein alone is used; in addition, animals so sensitized react positively to tuberculo-protein alone.

Every injection of either protein alone or with phosphatide is followed immediately by an outpouring of neutrophiles from the blood stream as well as by an increase in monocytes. Both of these types of cells appear in greater numbers after the mixed injections. However, it is the amount of the residual reactions of monocytes and epithelioid cells, also greater after the mixed injections, which correlates with the degree of sensitization.

Returning to the concept of Baldwin, that "if tuberculin reactivity is present in any animal, the presence of tubercles in that animal must be predicated," it is our opinion that it is possible to sensitize guinea pigs to active tuberculo-protein by virtue of the cellular reactions which this material induces. The protein itself can induce a new formation of monocytes and some preparations of the protein give rise to a few epithelioid cells. The use of the phosphatide with the protein greatly increases the formation of monocytes which become transformed into tubercle-like masses of epithelioid cells. We consider that it is this much increased cellular reaction due to the use of the phosphatide which, in the presence of the active antigenic agent, is correlated with the more rapid and the more effective sensitization. The use of the skin as the locus of the injection is also a factor since in this tissue the antigen remains concentrated for a longer time around the cells. The phosphatide plus protein used in the skin brings about a sensitization which is much like the sensitization of the disease itself. From these studies it seems justifiable to stress the fact that an increase in monocytes and epithelioid cells accompanies the phenomenon of sensitization and that in some way the phagocytic

mononuclear cells, specifically monocytes and their derivatives, epithelioid cells, play a definite rôle in the mechanism that sets up sensitization.

In the cellular reactions it is important to discriminate between the direct effects of the materials introduced and the indirect effects of the sensitization. The direct effects are the infiltrations with neutrophiles and the new formation of monocytes and epithelioid cells. There is also some infiltration of the lesions with eosinophiles certainly after the initial stages. Since they also occur after injections of phosphatide without sensitization, it is not possible at the present time to analyze their relation to sensitization. They have been noted repeatedly in sensitized tissues (Seibert, 8 a). It is interesting to note that we have not found any increase in lymphocytes in these dermal reactions.

The indirect effects of sensitization are edema, hemorrhage, and necrosis. The hemorrhage and necrosis set up new and complicated cellular reactions, new infiltrations with neutrophiles, phagocytosis of debris, and ultimately the new formation of vessels, new fibrous tissue, and bands of fibroblasts.

Our experiments indicate that the skin is a much more effective organ for inducing the type of sensitization that expresses itself in skin reactivity than either the lining of the peritoneal cavity or the subcutaneous tissue. This difference in effectiveness can be expressed by the following comparison. Using the peritoneal route of injection, the minimum amount of protein necessary to sensitize any guinea pig was 30 mg. (Seibert, 8 a); by the subcutaneous route, 5 mg. (Smithburn, Sabin, and Geiger, 19); by the intradermal route the amount can be reduced to 0.025 mg. when enhanced with phosphatide. These materials bring about the same cellular reactions whether introduced intraperitoneally, subcutaneously, or intradermally. After injection by the intraperitoneal route the induced cells are widely dispersed throughout the omentum and under the serosal lining of the abdominal viscera and parietal peritoneum. In the dermis, on the other hand, the reaction is limited to a small area, due to the fact that the injections are made into a dense feltwork of fibers which acts as a mechanical barrier to the spread of fluid. It is, of course, true that some of the injected fluid enters the dermal lymphatics immediately

and is carried to the regional lymph nodes, but there is also a drainage of fluid into lymph nodes from every zone of injection. With intradermal injections, however, the amount of protein remaining in a restricted area is much greater so that the effect of the unique structure of the skin as a sensitizing zone may be summed up in the phrase that it provides a greater "dose per cell" of the sensitizing agent.

The question must now be considered of whether the sensitization which can be induced by tuberculo-protein and enhanced by tuberculo-phosphatide, as evidenced by the intradermal test, is identical with the type of sensitization to foreign protein first described by Arthus, or is rather to be regarded as a special type to be known as tubercular allergy. If by Arthus phenomenon is meant a reaction in the skin of sensitized animals that runs somewhat parallel to the precipitin titer of the serum of the animal to the same antigen, and to the phenomenon of passive transfer by antibodies, then neither the sensitization in the disease tuberculosis, nor the sensitization induced by tuberculo-protein can be regarded as identical with the Arthus reaction. For Freund, Laidlaw, and Mansfield (24) have shown that in tuberculous rabbits there is no correlation between complement fixation and the skin test, and Seibert (8 *a, d, e*) has shown that the same is true of rabbits sensitized to the tuberculo-protein. By means of the use of tuberculo-phosphatide with protein, it is possible to induce in guinea pigs a sensitization manifested by a delayed reaction which appears in 24 hours, lasts for 48 hours, shows induration, hemorrhage, and necrosis, and is like a four plus tuberculin test in a tuberculous animal. When these animals are tested with 0.1 mg. of another preparation of protein (MA-100), they show characteristic three plus tuberculin reactions and four plus with necrosis when 0.5 mg. is used. It is our opinion that in the disease tuberculosis there is a mechanism of which some part, at least, is like an Arthus phenomenon; that in the disease the mechanism may be much more complex than is the reaction to a single protein, but that some of the difference may be due to a variation in the amount of antibody free in the circulation and in the amount and kind of change in the cells. If it be not legitimate to consider the change in the sensitized cells of the tuberculous animal as due to antibodies, it is, nevertheless, due to some change in the cells themselves, probably induced by proteins

set free from the infecting bacilli. That the cells themselves are sensitized to the protein was shown by Rich and Lewis (25) by the method of tissue culture. It was later shown by Moen (26) that the cells from a tuberculous animal remained sensitive after several generations in cultures.

Inasmuch as the animals highly sensitized to tuberculo-protein did not react to tuberculin, PPD, and did not show the Koch phenomenon when they were inoculated with living tubercle bacilli, it is clear that the changes in tuberculous animals are much more complex than in simple sensitization. The suggestion is made that the processes of immunization in tuberculosis are not identical with sensitization. Such highly sensitized animals offer valuable material for the further study of this relationship.

CONCLUSIONS

1. Guinea pigs can be rendered hypersensitive to tuberculo-protein by small, repeated, intradermal injections of active tuberculo-protein.

2. The addition of tuberculo-phosphatide to the protein speeds up the process of sensitization and enhances it so that the reactions become indurated and necrotic, closely simulating those of the disease.

3. Active tuberculo-proteins induce a new formation of monocytes and some epithelioid cells. The addition of phosphatide to the protein brings about a massive formation of epithelioid cells.

4. With the increased cellular reaction to the mixed injections may be correlated the increase in the speed and intensity of the sensitization.

5. The intradermal route is the best for these sensitizations, probably because it provides the greatest dose per cell of the sensitizing agent.

6. The degree of sensitization artificially obtainable by the synergistic action of tuberculo-phosphatide and tuberculo-protein is quite comparable to the degree of sensitization naturally occurring in tuberculous animals; moreover, this degree of sensitization may be induced with amounts of the materials from the bacilli which could conceivably be present in the tissues of an infected host.

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EXPLANATION OF PLATES

PLATE 29

FIG. 1. Site of the reaction 24 hours after an intradermal injection of 0.5 mg. of tuberculo-protein TPA (Seibert) in 0.1 cc. saline in a normal guinea pig (R 6292[3]). Figs. 1 to 8 are ¾ natural size.

FIG. 2. Site of the reaction 24 hours after the second injection of the same protein in guinea pig R 6200.

FIG. 3. Site of the reaction 24 hours after the third injection of the same protein in guinea pig R 6196.

FIG. 4. Site of the reaction 24 hours after the fourth injection of the same protein in guinea pig R 6198.

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[3] These numbers are serial numbers covering the work of the laboratory for a term of years.

(Sabin and Joyner: Tubercular allergy without infection)

PLATE 30

FIG. 5. Site of the reaction 24 hours after an intradermal injection of 0.5 mg. of tuberculo-protein TPA (Seibert) plus 5 mg. of tuberculo-phosphatide (Anderson) in 0.1 cc. saline in a normal guinea pig (R 6291).

FIG. 6. Site of the reaction 24 hours after the second injection of the same mixture, left side, with a flare-up of the first injection, right side, in guinea pig R 6192.

FIG. 7. Site of the reaction 24 hours after the third injection of the same mixture, upper right side, with a flare-up of the second, lower right, and of the first, left side, in guinea pig R 6188.

FIG. 8. Site of the reaction 24 hours after the fourth injection of the same mixture, showing massive necrosis, in guinea pig R 6191. The other three sites showed the flare-up but the photograph does not include them.

(Sabin and Joyner: Tubercular allergy without infection)

PLATE 31

FIG. 9. Section of the skin from the back of guinea pig R 6201, 24 hours after a first injection of 0.5 mg. of protein TPA (Seibert) in 0.1 cc. saline to show the increase in monocytes and neutrophiles in the lower part of the dermis and the subcutaneous tissue. Stained in Giemsa. × 375.

FIG. 10. Section of the skin from the back of guinea pig R 5790, 24 hours after a first injection of 0.5 mg. of protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline, to show the increased cellularity. Stained in Giemsa. × 45.

FIG. 11. Section of the area of densest cellularity of Fig. 10, showing a mixture of neutrophiles and monocytes in the deepest layers of the dermis. × 375.

FIG. 12. Section of the skin of the back of guinea pig R 6200-1, showing the site of a first injection of 0.5 mg. of tuberculo-protein in 0.1 cc. saline, taken 24 hours after a second injection had been made. This reaction is 7 days old. It shows an increase of monocytes in the dermis, especially marked in the perivascular areas. Stained in Giemsa. × 105.

FIG. 13. Section of the skin of the back of a guinea pig (R 6193-1), showing the site of a first injection of 0.5 mg. of tuberculo-protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline, taken 24 hours after the second injection had been made. This reaction is 7 days old. It shows tubercle-like masses of epithelioid cells in the dermis and in the subcutaneous level. Stained with Foot's modification of the Masson method. × 37.

FIG. 14. Section of a part of the tubercle shown in Fig. 5, showing the character of the epithelioid cells. × 700.

FIG. 15. Section from the skin of the back of a guinea pig (R 6192-3), 24 hours after a third injection of 0.5 mg. of protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline. The photograph shows young monocytes and eosinophiles beneath the carnosus muscle to which level the reaction had spread. Stained in Giemsa. × 700.
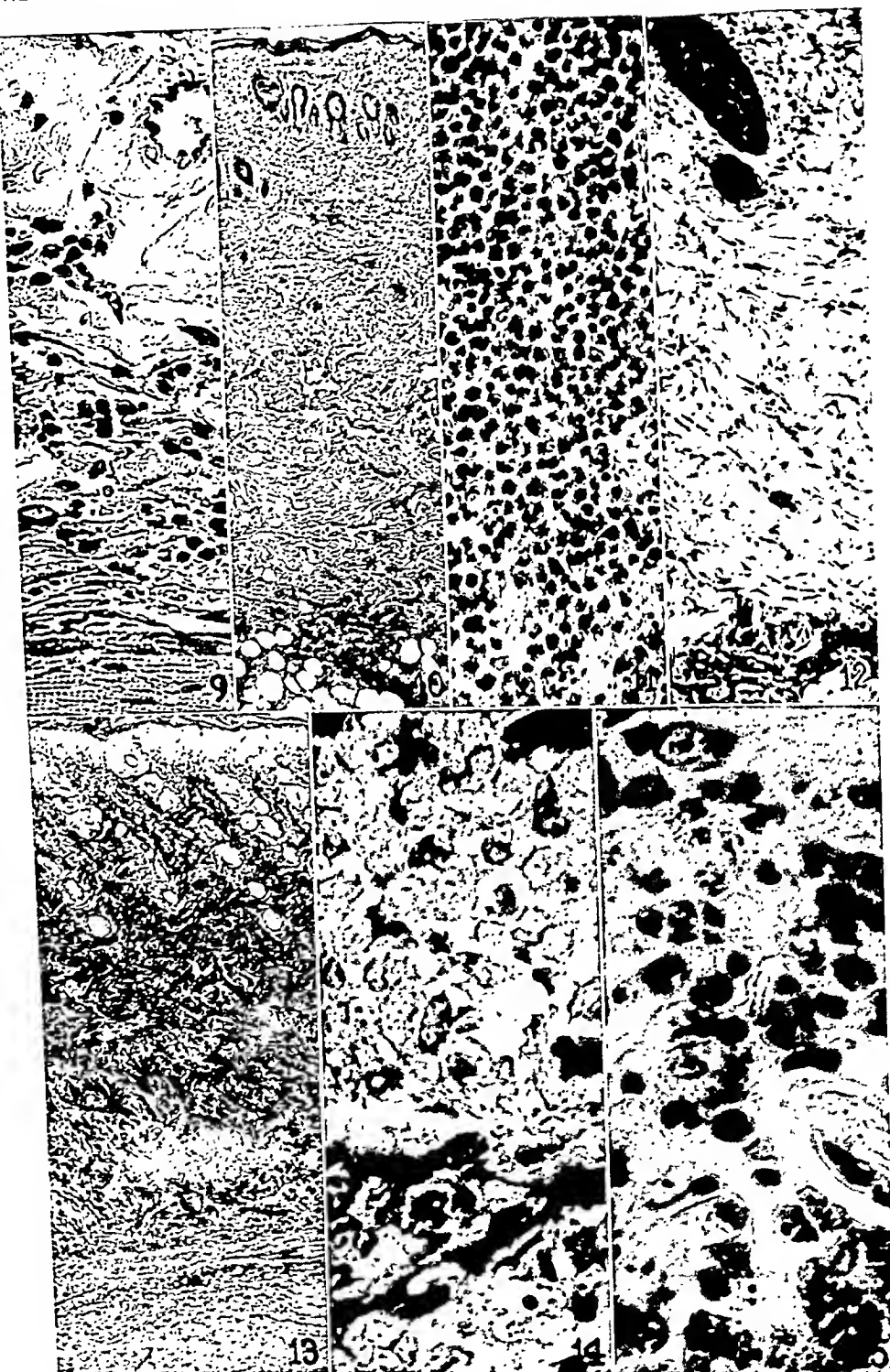
PLATE 31

(Sabin and Joyner: Tubercular allergy without infection)

STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

I. Histopathology of the Nervous System in the Guinea Pig

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Plates 32 to 35

The pathology of the nervous system in equine encephalomyelitis has been described for both the Western (1) and the Eastern (2) forms of the disease. Much attention has been paid to the intranuclear inclusion bodies, found in glial and mesodermal cells as well as in neurones. The intense inflammation, together with the necrosis of nerve cells, has been reported. It is the purpose of this paper to record in detail the neuropathologic features of the disease in the guinea pig, and to describe the histogenesis of the disease process. Only the nervous system findings are reported. Details of visceral pathology, described by Hurst (2), have been confirmed but no new observations added.

Material and Methods

In the experimental work a strain of the Eastern virus, isolated in the summer of 1937, was used. The virus was passed through guinea pigs, and the 2nd, 3rd, and 4th passages were used for subinoculations. It was considered especially important to keep the virus as close to its natural host as possible, for, as is well known, repeated passages through a new host may modify the characteristics of the virus. Guinea pigs were used exclusively, and were infected by a variety of routes, the principal methods being subcutaneously in the plantar tissues, intracerebrally, intraocularly, and intranasally. Animals were killed at various stages of the disease, the earliest being about 55 hours after inoculation, when symptoms were not yet manifest. The great majority of animals were perfused through the aorta with fixative before the brains were removed. Bouin's fluid, Heidenhain's Susa fixative, and 10 per cent formalin were the fixatives employed. Over 40 brains, all containing lesions, were sectioned serially in paraffin, at a thickness of 15μ. In most instances every 15th section was mounted and stained, but in

677

later cases, when the pathological findings had become thoroughly familiar, every 20th or even every 25th section was utilized. Phloxin- or eosin-methylene blue, hematoxylin and eosin, and thionin were used in different brains. Thionin was the most satisfactory. Some blocks, known to contain lesions, were sectioned serially, and every section mounted and stained. In this way the entire extent of the lesion was made available. In addition other brains, not cut serially, were examined in paraffin, frozen, or celloidin sections. Appropriate staining methods were used specifically for myelin, fat, and neurofibrils, as well as the usual cell stains.

OBSERVATIONS

Peripheral Inoculation.—Examination of the brain of an animal moribund from the disease shows a very extensive destructive process which may involve any or every part of the neuraxis. A great variety of pathologic changes may be observed. Since, however, the study of the late or end-stage teaches very little about the evolution or natural history of the disease, special attention has been paid to the very early stages. This term requires some definition. In an animal killed 55 to 60 hours after inoculation (which is 20 to 30 hours before the first symptoms become apparent), all lesions found may properly be called early. Such early lesions, however, vary greatly in intensity; that is, the observed abnormalities may be very slight or very marked. The damage is frequently very severe, but sharply limited and not extensive. In the later cases, killed after symptoms are well advanced, the difference from the earlier cases is largely quantitative; that is, lesions are more numerous rather than more intense.

From a study of a graded series of cases, a typical lesion may be described, occurring in both early and late cases. An example is illustrated in Fig. 1. There is a fairly well circumscribed inflammatory reaction involving a limited area of the cortex, tending to extend radially. In such a focus there are large numbers of polymorphonuclear leucocytes scattered through the tissue. In the molecular layer of the cortex the polymorphonuclear leucocytes are in general much fewer, and the infiltrating cells are composed chiefly of blood mononuclears and glial cells. Such an isolated focus may range in size from about 1.5 mm. to less than 0.5 mm. That is, in serial sections 0.225 mm. distant, a collection of leucocytes may be present through as many as 8 successive sections, or may be visible in only one section, with sections on either side completely normal. The overwhelming majority of lesions, however, involve more than one section. The leucocytes may vary greatly in number. Figs. 2 and 5, under higher

power, show average examples. In the more intense examples (as in Fig. 1), the center of the lesion shows a loss of all the nerve cells, with the destruction diminishing sharply towards the periphery. In less severe instances there may be practically no neuronal destruction, even though the leucocytes are no less numerous. In Fig. 5 the arrow points to a necrotic cell. In this, leucocytes may be seen (under the microscope) actually invading the cell body. The other neurones visible have normal nuclei and cell bodies, although this fact is not so well appreciated in the photograph as under the microscope where the focus may be altered.

The marked pyknosis is not the usual type of cell destruction in this disease. When neurones are damaged, the sequence of cell destruction is as follows: First apparent is a pallor of staining reaction, affecting both nucleus and cytoplasm, but cellular morphology remains completely unchanged. The cytoplasm gradually disintegrates, becoming more and more pale, and showing a foamy type of vacuolation and swelling reminiscent of the well known water-change artifact. The nucleus may be absolutely intact during this stage, even though the cytoplasm is disintegrating and the cell is surrounded by leucocytes. The cytoplasm may disappear except for a very fine peripheral rim, in the center of which is the pale nucleus. Forms may be seen where no cytoplasm is visible at all, while the characteristic nerve cell nucleus remains recognizable. In all these stages of cell degeneration the nucleus, though pale staining, shows a distinct membrane, within which is a pale granular reticulum. The nucleolus may or may not be well preserved. Finally even this disappears, and no trace is left of the nerve cell. · This type of change affects especially the granule cells of the cerebral cortex. The pyramidal cells exhibit to a greater degree a clustering of leucocytes around them, with vacuolation and granular disintegration of cytoplasm and nucleus occurring simultaneously.

The criterion of a necrotic nerve cell lies not in the condition of the cytoplasm but in the state of the nucleus. Where the architecture of the nucleus is preserved, that is, where the nuclear membrane is intact, the nucleolar complex normal, and the oxy- and basichromatin masses preserved, the neurone cannot be called necrotic. Severe cytoplasmic changes may, indeed, indicate irreversible changes, with the probability that the cell will soon die. But nuclear morphology, and not the tinctorial reaction of the cytoplasm, is the ultimate standard of the condition of the neurone. When eosin is used, a bright red staining of the cytoplasm, indicative of necrosis, is invariably accompanied by characteristic nuclear changes which are unmistakable and readily appreciated with thionin, or any other nuclear stain.

Figs. 6 and 7 show small circumscribed foci of polymorphonuclear leucocytes in the Ammon's horn and the cerebellar cortex, respectively. In Fig. 6 it should be especially noted that the pyramidal cells are entirely normal.

These foci, which may be large or small, dense or sparse, in early cases appear almost exclusively in the gray matter and chiefly in the neo- or the olfactory cortex.

Such non-cortical regions as the olfactory bulbs or basal ganglia, as well as the thalamus, are sometimes affected by similar circumscribed foci. These frequently show a relation to blood vessels but are in no sense perivascular.

Concomitant changes in the blood vessels are found. White blood cells, predominantly mononuclear elements, accumulate within the lumina. This fact is of especial significance when it is remembered that the vascular system is perfused before the brain is removed, and the vessels are empty of red blood cells. The leucocytes stick to the endothelium, undoubtedly through chemotaxis, and resist the washing-out action of fixing fluid pouring through the vessels. At the same time the endothelium is somewhat swollen and the adventitial cells hypertrophy markedly and proliferate, becoming, as well, strongly basophilic and hyperchromatic. Small spindle-shaped or moderately rounded forms predominate. The multiplication of adventitial cells is even more prominent than the collection of white blood cells in the perivascular space, that is, than the "round cell infiltration." If the focus is of any size the blood vessels invariably show this change. In examining the sections serially, the first sign of disorder will usually be this alteration in the blood vessels of a small area, observed for one or two sections without any parenchymatous reaction. Then for a section or two polymorphonuclears appear, and then the reaction disappears as further sections are examined.

The minimal lesion, that is, the least deviation from the normal that is recognizable as such, consists of this vascular change without any detectable alteration in the parenchyma. Figs. 3 and 4 illustrate this change. Fig. 3, under low power, is from the inferior colliculus. The affected blood vessels stand out vividly under low power. No change in the nerve cells or parenchyma is detectable. Fig. 4, from the caudate nucleus, and under higher magnification, shows more clearly the normal condition of the nerve cells. Exactly similar alterations are found in the cerebral cortex and other portions of the brain.

It must be emphasized that this change is not the "secondary" or "symptomatic" inflammation of Spielmeyer. It is a primary change, and the earliest reaction that has been observed. Only venules and arterioles are thus affected. Capillaries never showed demonstrable changes.

The regions where these early blood vessel changes are prominent, with or without a few polymorphonuclear leucocytes in the tissue, frequently show very fine inclusion bodies in the endothelium, vascular adventitia, and glial cells. Thionin, because of its metachromatic staining qualities, gives an adequate demonstration of such inclusions.

The more usual type of perivascular cuffing with lymphocytes and plasma cells also occurs in this disease, but chiefly in the more advanced cases. In the early lesions adventitial cell proliferation is the first reaction. In the more severe lesions, polymorphonuclear leucocytes are frequently found within the perivascular sheaths, a feature previously noted by Hurst. In advanced cases and even in intense lesions of early cases, such leucocytes have been seen infiltrating the walls of the blood vessels, so that the picture superficially resembles th...

Extravasation of red cells, sometimes restricted to the Virchow-Robin spaces, sometimes free in the tissue, is also of frequent occurrence. Hurst believed these to be artifacts subsequent to the trauma of removing the brain while the blood vessels were congested. But since they also occur when the brain has been perfused (under physiological pressures) and fixed before removal, such hemorrhages must be considered as antemortem findings.

Neuroglial reaction is prominent in this disease. Very early lesions may, atypically, show a glial mobilization in the affected region. Fig. 8 is a good example. In this field there is not a single polymorphonuclear leucocyte to be seen, although, several sections deeper into the lesion, a few make their appearance. Similar glial proliferation often occurs in the cerebellar cortex. Sections are available very similar to Fig. 7 but of about half the cell density, where no leucocytes are to be seen but where the proliferated cells are all glial. Diffuse glial increase is very frequently seen in the lamina zonalis of the cerebral cortex, where polymorphonuclear accumulations are the exception rather than the rule.

Some care must be exercised in designating a given nucleus as belonging to a glial cell. Among undoubted glial elements are fairly typical blood monocytes and large lymphocytes that on superficial examination may cause some confusion. These cells often occur in clusters. But similar types are often to be seen within the lumina of blood vessels or in the perivascular spaces.

The glial nodule (Fig. 12) is a somewhat different type of reaction. Such nodules were at one time thought to be peculiar to rabies, but are now known to occur in a variety of virus diseases. The typical closely packed cluster is not of frequent occurrence in the gray matter, and bears no necessary relation to the exudative changes. In the white matter, however, the nodule is a common finding, and is usually composed not only of glial cells, but also of blood mononuclears and even of some polymorphonuclears. Foci composed purely of polymorphonuclears do occur in the white matter, even in early cases, but are much less usual than the mixed focus or the pure glial nodule.

Although lesions in the gray matter may occur entirely at random, in the white matter the lesions are usually located where there is presumptive evidence of "nerve spread" of the virus. For example, with lesions occurring both in thalamic nuclei and in the appropriate cortical projection centers, the corresponding thalamic peduncle and the centrum ovale will often show the above mentioned type of lesion. Or when virus is introduced into the eye, and the optic centers are affected, the intermediate fiber tracts are also involved. In the white matter, however, the lesions are always discrete and discontinuous. In the gray matter, especially in the subcortical centers, an entire nucleus may be involved by the inflammatory process, but an entire fiber tract is never similarly affected. The foci are always spotty and frequently, though not always, in relation to blood vessels.

The foregoing description covers the very great majority of the lesions which occur after peripheral inoculation. They are obviously

inflammatory in nature, with mesodermal reaction (that is, intra- and perivascular or adventitial change) appearing first, with exudation the most prominent feature, and with demonstrable neuronal change clearly following and not preceding the onset of the process. More rarely, however, there are foci where severe degeneration or even total loss of nerve cells occurs in the absence of significant exudation. In the midst of normal cortical architecture there may be well defined regions where the neurones show all degrees of severe damage. Yet very few polymorphonuclear leucocytes may be present, and the degree of neuronal damage is disproportionate to the degree of inflammatory reaction. In such instances the term "degenerative reaction" may be employed to express contrast with the other more usual change previously described. A more striking example is shown in Fig. 14, where whole regions of Purkinje cells are wiped out. Here too a few polymorphonuclear leucocytes are visible under higher power, but the contrast with, for example, Figs. 1, 6, and 7, which are outspokenly inflammatory, is obvious.

Intracerebral Inoculation.—This distribution between inflammatory and degenerative is not meant to be an absolute dichotomy of the "either . . . or" variety. There is a certain degree of overlap. The value of this distinction, however, is immediately apparent when we consider, instead of peripherally inoculated animals, those which have received virus directly into the brain substance.

If the virus is injected intracerebrally, quite a different picture results. Clinically, the course of the disease is more rapid; with the dosage used, the virus placed in the brain substance causes death in 3 to 4 days, whereas a peripheral inoculation of comparable dosage requires 4 to 8 days for exitus.

The site of the inoculation into the brain is readily found in serial section. One of the most striking facts is that there is no special reaction, and no trace of virus encephalitis, around the needle track. Histologically the locus of injection does not differ at all from a needle wound with a bland non-specific injection mass. Polymorphonuclear leucocytes are not found, but only the familiar and quite characteristic glial reaction attendant on any needle puncture.

Yet the virus, introduced without causing especial damage, produces encephalitis throughout the entire brain. There is very widespre...

but relatively mild vascular and perivascular reaction, similar to that described for the peripheral type of inoculation but with much greater prominence of true round cell perivascular infiltration. However, after peripheral inoculation even very early cases show a much more severe focal reaction than is met with in intracerebral inoculation. Furthermore, in the latter cases, the perivascular hemorrhages are rare.

Although there are abundant polymorphonuclear leucocytes scattered through the tissue, they are more diffuse and rarely form such compact foci as with peripheral inoculation. The leucocytes appear singly or in small groups, and the dense accumulations, as seen in Fig. 1, for example, are most unusual in intracerebral injections.[1]

The constant and characteristic feature of intracerebral inoculation is the degenerative change mentioned above. That is, there is striking disproportion between the destruction of nerve cells and any inflammatory reaction that may be present. Fig. 13 shows a portion of the hippocampus with a stretch of the pyramidal cells simply wiped out. In this particular picture, a higher power examination shows a few leucocytes, but instances are available where there are none. Fig. 13 should be compared with Fig. 6, of the hippocampus after a peripheral inoculation. In the one there is a small focus of leucocytes, with intact neurones; in the other the destruction of ganglion cells is clearly the primary feature, the scattering of leucocytes secondary.

This type of involvement of the hippocampus is absolutely constant with intracerebral inoculation (it appears in all of 7 cases sectioned serially). Frequently the damage is exquisitely symmetrical, as shown by Fig. 9, where the exact segments destroyed on one side are equally destroyed on the other. (The inoculation, in this case, was entirely in the right hemisphere, as shown by serial section, and the needle track did not penetrate the ventricle.) Rarely, a very slight degree of this type of hippocampal damage is seen after pad inoculation, but only in cases of long duration, never in early cases.

Another type of destruction of nerve cells is seen in Fig. 10. The fundamental change may be seen at a glance. Whole massive areas of neurones are wiped out with scarcely a trace remaining. The glial cells suffer relatively little, and are present in essentially normal numbers. Leucocytes are scattered lightly through the tissue, without forming definite foci, but are no more frequent in areas where the neurones have vanished than where they remain intact.

These areas of cell loss are essentially random in distribution. The pyramidal cells are somewhat more severely affected, for, between the arrows, the external and internal granular layers are seen persisting rather feebly, although the pyram-

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[1] After intracerebral inoculation, severe involvement of the inferior olive is a quite constant exception to this statement.

idal cells of the 3rd and 5th layers are gone. At other times, however, this distinction does not obtain. The illustration here recorded is a particularly severe and widespread example. In other areas, and in other brains, the cell loss may be more patchy, wiping out an irregular area of neurones, but leaving the remainder of the lamination intact. Such *Verödungsherden* are frequently perivascular, and resemble the type of cell loss occasionally found in vascular disease or in some types of cerebral ischemia.

A further characteristic of the intracerebral inoculation is the severe degeneration found in the large multipolar ganglion cells of the brain stem, rarely found after peripheral inoculation with a long duration. Fig. 16, taken from the red nucleus, illustrates the type of change which corresponds to the "severe cell disease" (*schwere Zellerkrankung*) of Spielmeyer. The nuclei of the neurones remain intact, although they are somewhat eccentric; the cell bodies, though somewhat rounded, show relatively little change in contour. But the Nissl granules show profound changes, being reduced in some instances to a powder, with pale staining reaction, in other instances being only partially broken up and disarranged. The simple, relatively benign cell change of acute swelling with chromatolysis is not observed in this disease. The severe cell disease is much more ominous.

Especially to be noted is the fact that there is no inflammatory reaction around these affected neurones. They are being destroyed under the influence of the disease process. Yet, instead of the direct inflammatory change, seen in the earlier figures, the virus action is indirect or secondary. This point will be treated further in the discussion. Fig. 16 should be compared with Fig. 15 which is taken from an animal inoculated in the pads. Here the neurone is intact, as may be seen by comparison with other cells of the same nucleus (facial nucleus). Yet there is vigorous glial mobilization. On the other hand, in Fig. 16 the opposite obtains. In many of the affected large ganglion cells of the brain stem, and even in others which show but little morphological change, intranuclear inclusions are sometimes found. These are more prominent in the intracerebral than in the pad inoculations. This aspect is adequately described by Hurst.

Changes in the Spinal Fluid Pathway.—The involvement of the spinal fluid pathway deserves mention.

In early cases after peripheral inoculation there are scattered patches of meningeal exudate, chiefly with mononuclear cells. But almost invariably these areas are directly overlying a cortical focus of parenchymatous involvement. In favorable sections a cuffed blood vessel may be seen emptying into the subarachnoid space, and leucocytes packed along the sheath of this vessel are continuous with those in the pial meshwork. Occasionally, however, a small area of meningitis, mononuclear in type, may occur entirely independent of any parenchymal damage. Such independent meningeal foci, usually at the base of the brain, are not necessarily due to direct attack by virus discharged into the cerebrospinal

fluid. They may be non-specific in nature, although the alternative of virus etiology cannot be ruled out.

With intracerebral inoculation, wherein the virus comes into immediate contact with the subarachnoid fluid and its pathway, meningeal reaction is insignificant. Because of the presence of intranuclear inclusion bodies in cells of the pia-arachnoid, the susceptibility of meningeal cells to the virus is considered established. Yet the virus, which may produce such an intense reaction in brain tissue when injected peripherally, produces very little reaction when it comes into direct and immediate contact with the meninges through intracerebral inoculation. The reason for this peculiar behavior is not clear, but the fact is worth recording.

An interesting reaction is observed in the ependyma, in 20 to 25 per cent of cases. Fig. 17 illustrates the phenomenon, which has been found only in the lateral ventricles and may be either unilateral or bilateral. The ependymal cells have proliferated and have been thrown into folds, whorls, and small acini. Between the ependyma and the free surface of the ventricle there appear large numbers of interlacing spindle-shaped cells, apparently glial in nature. Leucocytes may or may not be present in the affected areas. This type of change has been observed as early as 58 hours after inoculation and may occur after either peripheral or intracerebral inoculation.

In Fig. 17, which comes from an advanced case, very widespread and intense inflammatory changes are observable. Superficial to the affected ependyma is a large dense focus of polymorphonuclear leucocytes, while elsewhere, in the cortex, centrum ovale, hippocampus, thalamus, and thalamic peduncle, various types of inflammation are visible. But there is no necessary relationship between the parenchymal inflammation and the ependymal change. The one may occur entirely without the other.

The ependymitis here illustrated and described is considered to be part of the pathology of the disease, although its fundamental nature is obscure. Conceivably it is not at all related to the virus action, but is merely fortuitous. However, this type of alteration has not been observed in this laboratory in guinea pigs not infected with equine encephalomyelitis.

The presence of leucocytes or red blood cells free in the ventricles or infiltrating the choroid plexuses, is a variable finding. In general, the longer the disease has progressed, the more such cells are found. Very definitely, however, the presence of inflammatory cells in the ventricles is not related to the involvement of the ependyma by an inflammatory focus. That is, probably the leucocytes do not invade the ventricular cavity from the side of the brain. One case serves as a vivid, but not the only, illustration. In an area of ependymitis a large focus of polymorphonuclear leucocytes extends from the white matter, through the ependyma and the spindle cells, to abut on the ventricular cavity. But the ventricle is free of leucocytes. In many instances, the presence of cells in the ventricular fluid appears to be roughly proportional to the degree of inflammatory involvement of the choroid plexuses, but this relationship is not absolute.

Myelin.—Myelin is not primarily affected by the disease process.

The guinea pig does not show spontaneous demyelination, nor have plaques of demyelination ever been produced experimentally as has been done, for example, with the cat, the dog, or the monkey. In equine encephalomyelitis, appropriate stains show that a considerable degree of inflammation may be present and yet the myelin sheaths are unaltered. However, areas do occur where the parenchyma is destroyed, and in such foci all tissue elements are severely injured. Myelinated fibers are no exception, and in areas of necrosis are seen to be lacking. The axis cylinders are more resistant to destruction than are the myelin sheaths, and in areas where the latter have completely disappeared, fragments of axis cylinders are still demonstrable by silver impregnations.

These areas of necrosis, however, are not productive of neutral fat. The inflammatory and abbau cells never include gitter cells, and specific fat stains have consistently failed to demonstrate free neutral fat in lesions. To eliminate the possibility of technical error, sections of canine disseminated encephalomyelitis were stained at the same time with affected guinea pig sections. The former readily showed free fat, the latter did not. This peculiarity of reaction of the guinea pig brain to the virus is more striking when mechanical injury is considered. For example, in intracerebral inoculation, the needle track may show numerous gitter cells. Thermal injury, performed in certain experiments to be published elsewhere, also showed an abundance of the compound granular corpuscles. But such cells have invariably been lacking in areas of necrosis produced by the virus, even though myelin has been destroyed.

One unusual type of myelin damage has been observed. In Fig. 11 the fiber tracts of the callosal radiation bordering the ventricle show a high degree of rarefaction, appearing to involve the axis cylinders as well as the myelin. The fibers have disappeared without a trace. There is a moderate diffuse infiltration with polymorphonuclear leucocytes and lymphocytes, but here too gitter cells are not present. The fibers of the centrum ovale, however, are quite normal. This type of damage is quite rare, but has been seen following both intracerebral and peripheral inoculation.

DISCUSSION

Throughout the foregoing description, emphasis has been placed on two different types of lesions. In the one the inflammatory reaction (that is, the exudation of leucocytes, and the alteration and proliferation in the mesodermal vascular adventitia) is prominent. In the early examples, neuronal damage may be entirely non-existent; or, later, neurones may show varying degrees of damage more or less proportional to the degree of exudation. In the second type, neuronal degeneration or destruction is marked, while the inflammatory changes are insignificant or even entirely lacking. These

two types, however, are not mutually exclusive, yet for the sake of convenience the reactions may be called inflammatory and degenerative, respectively.

Following peripheral inoculation of the virus, in the overwhelming majority of lesions, the inflammatory reaction appears in scattered cerebral foci. In the earliest detectable cases, alterations are visible only in the lumen and adventitia of the blood vessels, with the nerve cells completely intact. From this beginning, graded transitions to the most profound inflammatory lesions may easily be traced. The degenerative reaction, that is, destruction with relatively insignificant amount of exudation, sometimes occurs but is not prominent. Following intracerebral inoculation, on the other hand, there is profound nerve cell destruction, while the inflammatory changes are, with few exceptions, slight.

Hurst (2), in his study of the histopathology of this disease, stated that the lesions were substantially the same in the two routes of infection, although they were more intense in the animals inoculated peripherally. This he attributed to the longer duration of the disease in such cases, with longer time for the changes to develop. Similarly, Syverton (3), when the present data were presented before the American Association of Pathologists and Bacteriologists, raised the question whether the difference between the two types might not be explained on the time factor.

This is clearly not the case. Animals killed 58 hours after subcutaneous injection in the pad may show inflammatory changes of extreme severity. Here the virus must first have multiplied locally in the leg, then been transferred to the brain. The virus has thus been present in the brain for much less than the 58 hours. On the other hand, direct injection into the brain, with sacrifice 67 to 78 hours later, has given much more time for the virus to act. Yet the lesions are quite different. Since the time factor cannot be invoked as the explanation of the difference, we are forced to conclude that we are dealing with two types of reactions that are perhaps fundamentally distinct.

There exists a certain amount of confusion around the problem of necrosis of nerve cells, and the question of whether neuronal damage precedes or follows the inflammatory reaction. The simple generalization of a picture of events,

stated and implied in many writings of Hurst and coworkers, might be given as follows: Neurotropic viruses, as obligatory intracellular parasites, invade the neurone (usually by way of the axis cylinder) and by primary and direct attack cause its destruction. If the animal be killed at a suitable stage (4) the ruling picture will be acute necrosis of ganglion cells. But the animal may survive longer than this initial moment, "and then, as in all lesions accompanied by necrosis of tissue, polymorphonuclear leucocytes migrate swiftly into the damaged areas" (5). "While, therefore, degeneration in nerve cells and perhaps in glial cells is clearly primary, and at an early stage in some localities of the nervous system may occur in the absence of any trace of inflammation in the mesodermal structure, this latter phenomenon is an integral part of the later histological picture." It is in accordance with this view that the distinction between intracerebral and peripheral inoculation is minimized, the former being taken to represent the acute stage, before inflammation has developed, wherein necrosis of cells is the ruling picture, while the latter represents the late stage.

With the foregoing viewpoint we find it impossible to agree entirely, for the following reasons.

1. It is erroneous to maintain that exudation of polymorphonuclear leucocytes is the natural reaction to necrosis of nerve cells *per se*. In cases of non-infectious etiology, as well as in certain cases of non-virus infections, many instances may be found of selective neuronal necrosis without exudation of polymorphonuclear leucocytes. A few of the most striking examples are: destruction of the hippocampal pyramidal cells, especially in the Sommer's sector; selective destruction of the Purkinje cells of the cerebellar cortex in many toxic conditions (6, 7); and the *Verödungsherden* of the cerebral cortex, chiefly ischemic in origin. Glial reaction may be present, and even perivascular round cell accumulation, but not polymorphonuclear leucocytes.

2. Recent research (8–10) has strengthened the older idea that polymorphonuclear leucocyte exudation represents a specific chemotactic response to specific agents. These agents are probably multiple, and either exogenous or endogenous in nature. It is conceivable, though unlikely, that virus particles are in themselves intrinsically chemotactic. It is more likely that virus action on the tissue produces intermediate products which are chemotactic and responsible for the exudation.

3. The earliest demonstrable changes are the mesodermal reactions described previously, which are antecedent to the passage of leucocytes into the tissue. Later, abundant examples are available that polymorphonuclear leucocytes invade the parenchyma in the absence of neuronal necrosis.

4. The presence of frank inflammatory changes with little or no neuronal loss, and the presence of extensive neuronal destruction with little or no inflammatory reaction, indicate that two distinct factors (or chains of factors) are operative. The time factor, once it is actually measured in hours, is seen to be irrelevant. The contention that degeneration (neuronal destruction) is primary, while the inflammatory reaction is secondary, is not in conformity with the present data. Rather would these two elements seem to be coequal in importance, sometimes

acting together, sometimes separately. With peripheral inoculation the inflammatory component predominates; with intracerebral inoculation, the degenerative.

It is here maintained that the factors responsible for the inflammatory and degenerative components are qualitatively and not merely quantitatively different. Thus, it is claimed that on a basis of simply "more" or "less," Fig. 1 could never develop from Fig. 10, nor Figs. 6 and 7 from 13 and 14, respectively. Qualitative differences must enter in.

In a tentative fashion the following hypothesis may be suggested. To a certain extent the destruction of nerve cells is a non-specific effect of the disease. That is, although the virus is the inciting agent, it is not the primary and direct causative agent. Rather, its effect is produced by certain intermediate steps which may be common to a wide variety of conditions producing a similar end-result. For example, the symmetrical necrosis of the Ammon's horn (Fig. 9), although in this instance caused by the virus, is quite similar to the picture found in epilepsy, general paresis, or insulin shock. Perhaps some common factor mediates the destruction, this common factor being in turn produced by a variety of conditions. Likewise, the destruction of the Purkinje cells (Fig. 14) is similar to that found in other non-virus diseases. Where many widely dissimilar conditions produce a more or less common effect, none of the conditions in question is the primary and direct cause of the result. More likely, all these dissimilar conditions have some factor in common, acting on tissue which is especially vulnerable.

There is no intention of setting up here a rigid scheme of dichotomy. The factors at work in disease are multiple and overlapping. It is necessary to point out, however, that destruction of tissue is not a unitary concept. If elements are destroyed by one mode, one type of histological reaction will result; another mode will produce a different picture. The presence or absence of polymorphonuclear leucocytes, of neutral fat, of gitter cells, or lymphocytes, all indicate differences in the mode of action, referable, probably, to different intermediate steps. Just how any virus works is at present unknown. In part it may act primarily on nerve cells; in large part, however, the primary action is interstitial. Or, in part, some of its apparent action on nerve cells may be secondary and indirect, due to special vulnerability of these elements to intermediary factors resulting from virus activity.

It is impossible, in the present state of our knowledge, to disentangle these threads without too many groundless hypotheses. It must suffice, at present, to lodge a protest against any view of pathogenesis which is too schematic or oversimplified.

SUMMARY

The action of the virus of equine encephalomyelitis in the guinea pig brain has been studied, and various histological changes have been described in detail. After peripheral inoculation (as in the pad) the earliest detectable pathologic change in the nervous system is the accumulation of leucocytes within the lumen of blood vessels, and the proliferation of the vascular adventitia. This precedes the appearance of any significant perivascular cuffing, and may or may not be accompanied by a few polymorphonuclear leucocytes in the tissue.

The typical lesion is a fairly well circumscribed focus of polymorphonuclear leucocytes accompanying the blood vessel changes described above. The leucocytes may be numerous or sparse, and may or may not be accompanied by neuronal destruction.

In early cases, before the onset of symptoms, such circumscribed lesions appear in small number irregularly scattered through the gray matter. The neo- and olfactory cortices are the principal sites of predilection, although basal ganglia, thalamus, cerebellum, and lower olfactory centers may also be involved. The hippocampus is much less affected than other parts of the brain.

A rough distinction is made between inflammatory and degenerative lesions, a distinction which depends on the relationship between the neuronal destruction and the exudative changes in any given site. These two types are described, and their significance is discussed.

After intracerebral inoculation, the inflammatory changes are much less marked than after peripheral inoculation. This is due not to insufficient time for the development of lesions but to a different type of pathological process.

Following intracerebral inoculation, there is primary destruction of neurones, involving especially the hippocampus, and also large areas of the neo-cortex. This change, similar to ischemic necrosis, is regarded in part as a non-specific reaction of especially vulnerable tissue.

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EXPLANATION OF PLATES

All figures are unretouched photographs of sections 15μ thick.

PLATE 32

FIG. 1. Typical encephalitic focus, with prominence of blood vessels, extensive leucocytic infiltration, and mild overlying meningeal reaction. The focus extends radially the width of the cortex. Pad inoculation. Thionin. × 32.

FIG. 2. Higher power photograph of a focus similar to Fig. 1. The abundant polymorphonuclear leucocytes stain rather weakly. The neuronal architecture is not disturbed, and the intact neurones are readily appreciated. Nuclei are perfectly normal. In the upper part of the figure are a few dark staining neurones, which, although hyperchromatic, are normal for this cortical area. Pad inoculation. Thionin. × 127.5.

FIG. 3. A field from the inferior colliculus, showing the minimal degree of detectable pathology. The three blood vessels indicated by arrows show proliferation of the vascular adventitia, but no round cell cuffing. Within the lumina, numerous white blood cells are adhering to the endothelium. There is no nerve cell destruction. Pad inoculation. Thionin. × 32.

FIG. 4. A similar lesion, from the caudate nucleus, bordering the ependyma. The nerve cells are entirely intact. Pad inoculation. Thionin. × 82.5.

PLATE 33

FIG. 5. Another cortical focus, similar to Fig. 2. This section is a little more deeply stained. Neurones morphologically normal and with intact nuclei are present among the very dense collections of leucocytes. The arrow points to a single neurone which is being invaded by polymorphonuclear leucocytes. The dark staining cell above it is perfectly normal, for the nucleus can be clearly seen under the microscope by slightly altering the plane of focus. Pad inoculation. Thionin. × 151.

FIG. 6. Hippocampus, showing a circumscribed focus of polymorphonuclear leucocytes. The pyramidal cells are normal. Pad inoculation. Thionin. × 115.

FIG. 7. A similar type of lesion, but located in the molecular layer of the cerebellar cortex. There is moderate meningeal reaction in the sulcus overlying the lesion. Pad inoculation. Thionin. × 39.

FIG. 8. Early focus in the cerebral cortex. There is an increase in glial cells in radial streaks, indicated by arrows. No polymorphonuclear leucocytes are present in the tissue. There is a moderate degree of adventitial hypertrophy in the blood vessels. The neurones are normal. The dark streak at the surface of the brain represents not meningitis, but a thin layer of India ink applied to the tissue before section to indicate the right side of the brain. Pad inoculation. Thionin. × 50.

(King: Pathology of equine encephalomyelitis)

PLATE 34

FIG. 9. Intracerebral inoculation. There is bilaterally symmetrical necrosis of the pyramidal cells of the hippocampus, indicated by arrows. There are a few polymorphonuclear leucocytes free in the neocortex, and a very minute degree of perivascular cuffing, neither of which is appreciable at this magnification. The contrast with Fig. 1 is readily apparent. Hematoxylin-eosin. × 7.

FIG. 10. Intracerebral inoculation. Massive areas of neuronal disappearance in the neocortex. The retrosplenial cortex, at the upper right, is normal. Between the arrows is an area where the 2nd and 4th layers of the cortex are better preserved than the 3rd, 5th, and 6th. At the left, however, all layers are equally affected. The dark line on the surface again represents India ink, and not meningitis. The absence of significant inflammatory changes should be noted. Thionin. × 15.

FIG. 11. Rarefaction of myelinated fibers of the callosal radiation. The caudate nucleus is in the upper right. The ependyma bordering the injured fibers is destroyed. In the rarefied area there is an increase in cells, but no gitter cells or compound granular corpuscles are present. Intracerebral inoculation. Hematoxylin-eosin. × 95.

FIG. 12. Isolated glial nodule in the neocortex, without inflammatory changes. Cortical architecture undisturbed. Pad inoculation. Thionin. × 54.

PLATE 34

9

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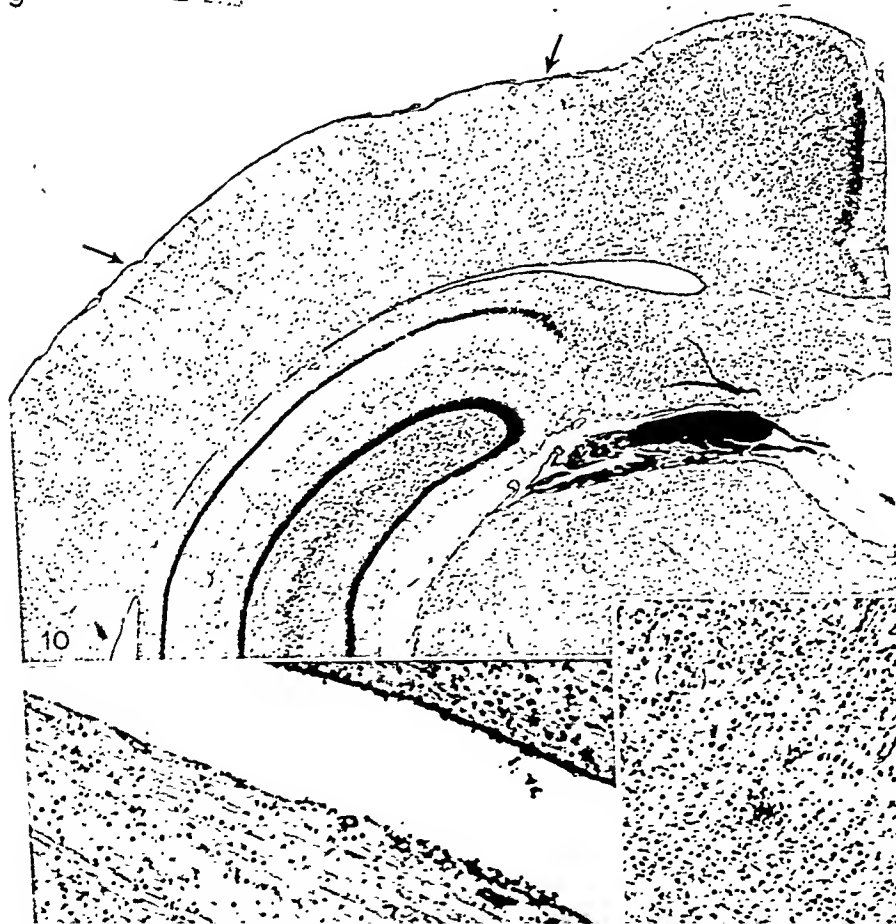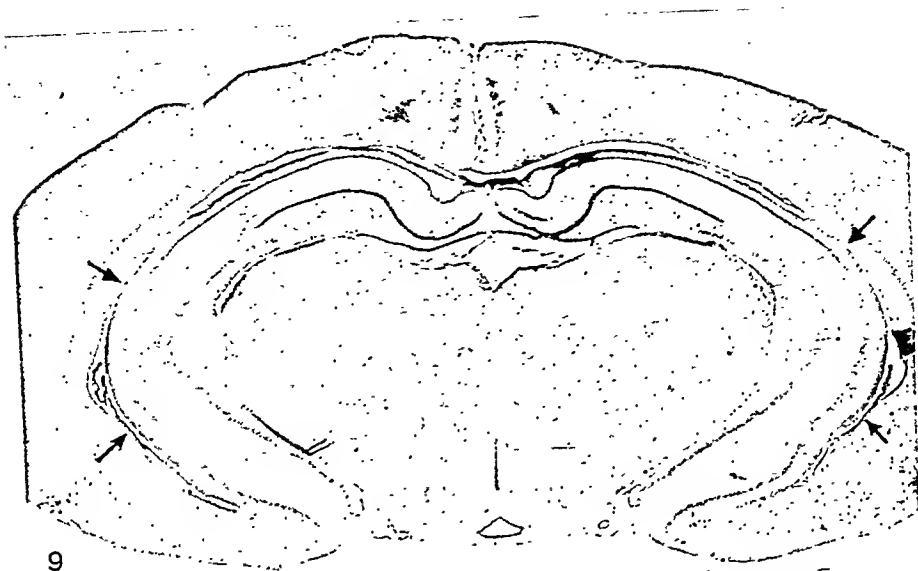
PLATE 35

FIG. 13. Hippocampal necrosis following intracerebral inoculation. This picture should be compared with Fig. 6, showing the reaction after pad inoculation. Thionin. × 87.

FIG. 14. Cerebellar cortex, illustrating the destruction of Purkinje cells in the absence of significant inflammatory reaction. This should be compared with Fig. 7. Pad inoculation. Phloxin-methylene blue. × 40.

FIG. 15. Clustering of glial cells around a morphologically intact neurone from the facial nucleus. Pad inoculation. Phloxin-methylene blue. × 157.

FIG. 16. Severe degeneration of neurones in the red nucleus. Intracerebral inoculation. Thionin. × 227.

FIG. 17. Ependymitis of the lateral ventricle. In addition, intense inflammatory changes are present throughout. Pad inoculation. Phloxin-methylene blue. × 64.

PLATE 35

THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. 63
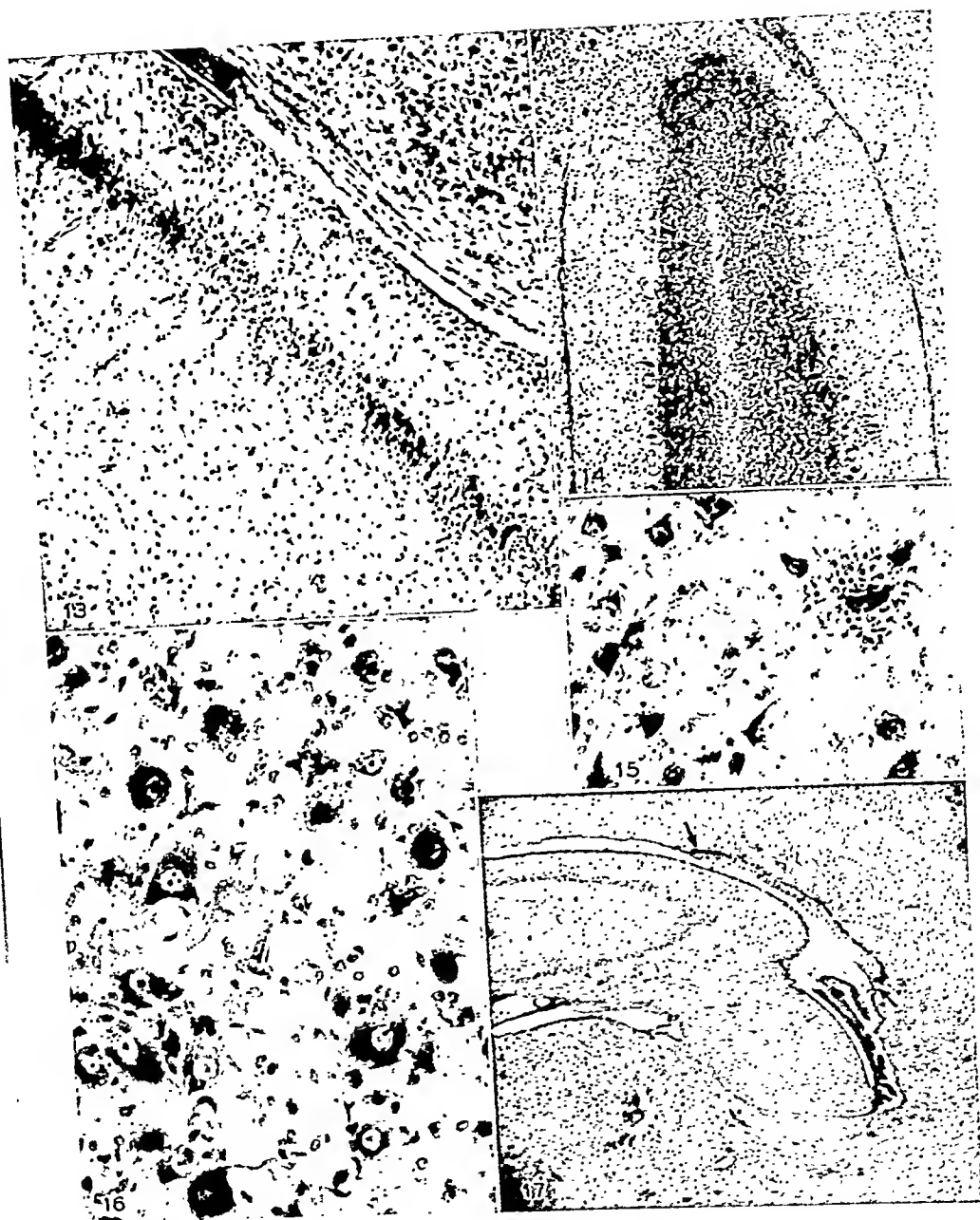
(King: Pathology of equine encephalomyelitis)

THE PRODUCTION BY A NEW METHOD OF RENAL INSUFFICIENCY AND HYPERTENSION IN THE RABBIT*

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The method described in the present paper was devised to make possible the production of renal insufficiency of any desired grade and without pathological changes in the kidney. Renal insufficiency has been produced by a great variety of harmful mechanisms such as renal poisons, bacterial agents, and x-rays, but a desired degree of insufficiency cannot be easily obtained with these procedures and the kidneys always undergo a profound pathological change. Surgical removal of one kidney with partial ablation of the other is more satisfactory, but is complicated by the hypertrophy which develops in the remaining tissue, and besides there is always a considerable amount of damaged tissue bordering the new surfaces created by the surgery.

A note has already been published on the method (1), but since then a few changes have been introduced.

Method

A loop of fine silk thread is placed around the left renal artery of the rabbit when it is small (400 gm. or less). The left side is chosen because of its accessibility. The diameter of the loop can be accurately adjusted by tying the thread down on the artery and a wire which itself has a measured diameter and is placed alongside the artery. The tie is made snugly but not tightly, so that the lumen of the artery is occluded by the tie, but the wall of the artery is not crushed or damaged in any way. The wire is then withdrawn from the loop and the artery is left with the loop around it. The size of the wire should be such that the loop

should have a somewhat larger diameter than the artery. The artery soon grows up to this size and then any increase in the blood flow and hence growth of the kidney, is prevented. The other kidney overdevelops and so takes care of the increased renal demands of the animal. When the animal is 3 to 4 months of age this large kidney is removed, leaving the animal with only the small kidney, in which hypertrophy is hindered by the restricted blood flow.

The purpose of using small rabbits and putting loops slightly larger than the renal arteries around these vessels, was to avoid any possibility of injuring kidney tissue by an inadequate blood supply. By allowing the renal artery to grow up to the size of the loop, the kidney is allowed to adjust its size to the blood supply and will grow no larger than is proper for its circulation. This point is particularly important in working with the rabbit. If the blood supply to the kidney is cut down from what is normal for a given size of the organ, necrosis usually develops with rapid disappearance of the organ. Thus a loop of diameter 0.5 mm. placed in a small rabbit will allow the kidney to grow up to a length of around 30 mm. while the animal is growing to maturity (1500 to 2000 gm. body weight). When a loop of this size is placed around the renal artery of a rabbit of this weight it almost always results in necrosis and resorption of the kidney. This was the experience with 5 out of 6 rabbits so treated. The same result was obtained with loops of 0.63 mm. diameter. Even when the loop is made 0.8 mm. (quite a large diameter for the rabbit) one gets a high percentage of necrotic kidneys. In 15 adult rabbits tied off in this way 6 kidneys became necrotic.

Orienting Experiments

In the first experiments different size wires were tried out to find the most favorable diameter for the loop around the artery. Four groups of 6 rabbits each were used. At a time when they were about 200 gm. in weight loops of diameters of 0.41, 0.53, 0.63, and 0.8 mm. were laid around the left renal arteries in the respective groups. The animals were allowed to grow for 3 months at which time they had body weights ranging around 1500 gm. The right kidneys were taken out at this time.

All rabbits in the group that had the left renal artery tied to 0.41 mm. died within a few days. The non-protein nitrogen was 129 in one of these on the 3rd day after removal of the right kidney. The average kidney weights were right side 5.8 gm., left 3.6 gm. In the group in which the artery was tied to 0.53 mm. the kidneys were the same size on both sides when the right was taken out. These animals survived for longer periods but with evidence of diminished renal function indicated by cessation of increase of body weight, and increase in blood non-protein nitrogen. The group with the artery tied to 0.63 mm. showed no difference in size of the kidneys and less

evidence of diminished renal function after operation, and the group tied to 0.8 mm. showed no evidence of diminished renal function after removal of the right kidney. We therefore chose the diameter 0.53 mm. as that most likely to curtail definitely kidney function but still allow the animal to survive long enough so it could be studied.

After having settled on the most effective diameter for the arterial loop the next question which is to be answered is the proper amount of time to allow before taking out the normal kidney. The left kidney grows until its length is about 3 cm. and then remains at this size. The body continues to grow, and this requires increase in the renal tissue which is taken care of by growth of the right kidney. The kidneys of the rabbit can be easily palpated through the anterior abdominal wall, which is quite thin, and one can estimate the length of the organ quite accurately by placing one's fingers at either pole and measuring with calipers the distance between them. The longer one waits then, the smaller becomes the ratio of the weight of the left kidney to the total kidney tissue weight. Consequently the longer one waits before taking out the right kidney, the greater will be the degree of renal insufficiency after the removal. Furthermore if one waits more than 2 months after the left kidney has become stationary one will often find connective tissue infiltration of this kidney, and still later, atrophy. It is of interest then to determine the proper ratio—left kidney weight to total kidney weight—to wait for before right kidney removal, in order to get the proper degree of renal insufficiency. Naturally the degree of renal insufficiency desired depends on the problem that one wishes to study and consequently the entire results are given as they occurred. In Table I are given the results of a series of animals in which the weight of the left kidney at operation varied from 20 to 48 per cent of the total kidney weight. The data given in this table are: body weight at operation, length and weight of right kidney (after excision), length of left kidney at operation, and the weight of the left kidney at the time of operation. This last figure naturally had to be estimated. If the survival time was short and at autopsy it was found that the left kidney length was the same as at operation the weight of the kidney at autopsy was used for the weight of the kidney at operation. If the rabbit survived for some time and the length of the kidney increased after

TABLE I

Summary of results in rabbits in which renal insufficiency was induced by nephrectomy of the normal right kidney from animals that had the growth of the left kidney restricted by a loop around its artery. This table gives the relationship between the ratio: $\dfrac{\text{weight of left kidney at nephrectomy}}{\text{weight of total kidney tissue at nephrectomy}}$ and the survival after nephrectomy.

Rabbit No.	Body weight	Right kidney length	Left kidney length	Right kidney weight	Left kidney weight	Left kidney weight / Total kidney weight	Survival time
	gm.	mm.	mm.	gm.	gm.	per cent	days
1	2510	42	25	13.0	3.3	20	2
2	2160	36	22	11.6	3.0	21	6
3	3240	40	27	15.5	4.0	21	8
4	2630	40	24	12.5	3.5	22	3
5		35	22	9.0	2.5	22	9
6	3415	45	26	11.2	4.0	26	7
7	2115	39	21	12.0	4.4	27	5
8	2440	39	26	12.5	5.0	29	6
9	2110	37	29	10.5	4.2	29	10
10	2735	44	30	16.5	6.5	30	5
11	3105	40	28	11.7	(5.2)	31	56
12	2200	35	27	10.0	(4.6)	32	37
13	3720	41	29	12.5	(5.8)	32	28
14	2245	39	28	11.3	(5.2)	32	19
15	3570	42	33	15.9	(8.0)	33	42
16	2770	42	32	14.5	7.0	33	43
17	2875	44	35	15.5	8.0	34	1
18		39	32	14.0	(7.5)	35	305 (killed)
19	2155	35	29	10.0	5.7	36	7
20	2240	37	30	9.5	5.5	37	6
21	2635	42	34	13.5	8.8	39	28
22	1625	32	28	7.7	(5.2)	40	21
23	2470	42	38	13.5	9.0	40	23
24	2635	37	33	11.0	(8.0)	42	140 (living)
25	2210	40	32	11.0	8.0	42	7
26	1705	39	32	9.9	7.5	43	9
27		38	35	12.5	(9.3)	43	153 (killed)
28	2895	36	32	9.0	(7.5)	45	90
29	1925	39	31	8.5	(6.9)	45	125
30	2950	37	34	10.5	(8.8)	46	115
31	1715	34	32	8.0	(7.5)	48	115

operation, the weight of kidney at the time of operation was estimated from its length. For this, use was made of a chart in which all the kidneys of the series were plotted with respect to weight and length. Figures so obtained are given in parentheses. The next figure in the table is the percentage that the left kidney weight bears to the total kidney weight at operation. The last column gives the survival time in days. It is apparent that if the left kidney weighs less than 30 per cent of the total at the time of right kidney removal severe renal insufficiency is to be expected with a very short survival time. If it is between 30 and 40 per cent of the total weight, serious insufficiency is to be expected although many cases will survive for periods sufficient for some limited study of them. However, if one wants a preparation to study for an extended period it is best to have the left kidney be between 40 and 50 per cent of the total kidney tissue at the time of operation.

Course after Production of Renal Insufficiency

The rabbits after the unilateral nephrectomy excrete urine. The quantity in general is greater, the greater the ratio—left kidney weight to total kidney weight. For those that had a ratio between 20 and 30 per cent the volume of urine per day varied between 30 cc. and 80 cc. and the specific gravity between 1.010 and 1.020. For those with ratios above 30 per cent the volumes per day were between 60 and 150 cc. per day with specific gravity usually above 1.020 and sometimes as high as 1.035. 2 rabbits secreted large amounts (400 to 600 cc. per day) of low specific gravity (1.005). It is of interest to note that those animals in the 30 and 40 per cent groups that died in 7 to 10 days had urine volumes as high as the others in this group and urine specific gravities above 1.020. All urines tested have shown a faint trace of albumin but never any more than that.

Practically all rabbits showed a steady loss of weight if the reduction in kidney tissue was large. The animals ate nothing or very little, indeed in some cases it seemed as though death was due to nutritional insufficiency: thus rabbit 16 went down in weight from 2770 gm. to 1550 gm. in 43 days. Rabbit 9 went down from 2110 to 1445 gm. in 10 days. Even when renal insufficiency was not so great

and the animals survived for a long period there was not the normal increase in weight but rather a constant weight or slight decrease. A frequent finding in these animals is a disturbance in the equilibrium apparatus. They can sit quietly without trouble but when they move about, or are moved passively, they make exaggerated back and forth lateral movements, or rotary movements, of the head. In righting themselves from the lying to the sitting position, they make very violent and exaggerated movements.
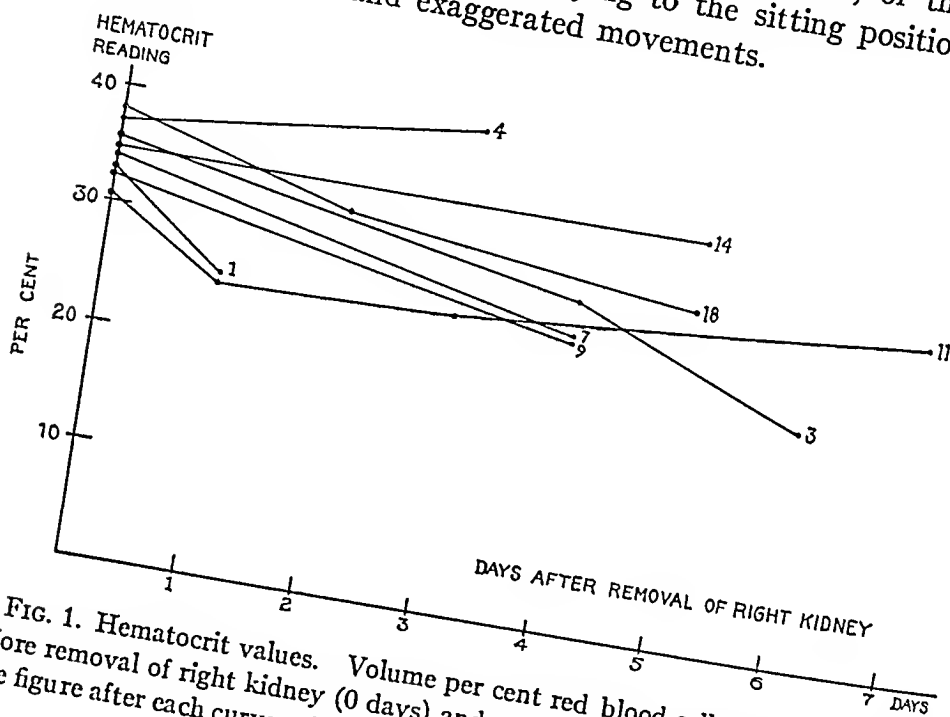
FIG. 1. Hematocrit values. Volume per cent red blood cells in the blood just before removal of right kidney (0 days) and at various times, in days, thereafter. The figure after each curve refers to the number of the rabbit as given in Table I.

A very frequent finding at autopsy was hemorrhages in the intestinal tract. The bleeding was in the walls of the intestine so that they had a blotchy appearance when viewed *in situ*. On opening the gut frequent submucosal hemorrhages would be apparent and bleeding into the lumen of the gut was quite frequent. In one case, rabbit 14, there was a pronounced hemorrhage into the eyeball. Anemia was a very frequent finding in these animals. The hematocrit value for a group of these rabbits, before and after operation, is shown in Fig. 1. In one case the reading went up slightly, in one it remained level, and in all others it went down definitely. Judging

from the results obtained on those in which readings were made a day or two after the operation, this anemia develops very rapidly. In two cases the hematocrit was followed for longer periods than shown in the chart. Weekly estimations were continued on rabbit 11 up to death, and except for the one reading of 28.4 per cent, shown in the figure on the 7th day, the values were consistently around 25 per cent compared with the preoperative 30.1 per cent. Rabbit 18 maintained a level between 28 and 30 per cent for 6 weeks after operation compared with the preoperative value of 38 per cent.

In 9 of the animals the blood pressure was followed. It was determined by placing the ordinary clinical sphygmomanometer cuff around the lower abdomen of the rabbit and the pressure determined just as in man except for using the abdominal aorta instead of the brachial for ausculation. In all cases, both before and after the nephrectomy, the blood pressure was above the normal (120/90). The pressure always rose after the right kidney removal except for one case in which it remained the same. The pressure before nephrectomy averaged systolic 194, diastolic 142, with the upper and lower limits of 230 and 160 for systolic and 185 and 110 for diastolic. After right kidney removal the average systolic was 222 (limits 260 and 185 and average diastolic 169 with limits 210 and 115). The hearts, ventricular tissue only, were weighed and the weight was divided by the gross body weight at autopsy to get the heart weight per kilo. The average for these values was 3.89 gm. heart per kilo body weight. The mean value for normal rabbits is given by Brown, Pearce, and Van Allen (2) as 2.35 gm. heart (including auricles) per kilo body weight. Our values are probably somewhat raised on account of the low food intake, but this is to some extent counterbalanced by the fact that we did not include the auricles in our heart weight. One may conclude that the heart weight is definitely above normal in this series and this agrees with the high blood pressure shown throughout. It will be noted that the blood pressure is elevated before operation, when the hypertrophied right kidney is functioning and there is no renal insufficiency. This confirms, in rabbits, the finding of Goldblatt (3) in dogs, that ischemia of one kidney will produce hypertension even though the other is intact.

Some experiments were carried out in the attempt to produce hyper-

tension in adult rabbits without a reduction of total kidney function. All rabbits in which a loop of 0.5 mm. had been laid around the renal artery when they were small show a definite hypertension by the time they are grown even before the reduction of total kidney mass. As explained earlier tying of the renal artery to 0.5 mm. in the adult practically always leads to kidney necrosis. In the 15 rabbits referred to earlier in which the right renal artery was tied to 0.8 mm. when the animals were already adult, 6 kidneys became necrotic. Of the 9 remaining 6 developed hypertension of a moderate degree: average systolic 172, limits 200 and 160; average diastolic 118, limits 150 and 100. 3 of the rabbits did not develop hypertension. The right kidneys of these were then removed whereupon they all developed a rise in blood pressure to 180/140, 200/120, and 165/135. One can conclude then that if one wishes to produce high blood pressure in rabbits already adult, tying one renal artery to 0.8 mm. is the best procedure. This will produce a fair degree of hypertension, which can be enhanced by removal of the other kidney. To produce more severe grades of hypertension one should tie the left renal artery to 0.5 mm. when the rabbit is small and remove the right kidney when the animal grows up.

DISCUSSION

There is evidently a marked difference in the ability of the kidneys of different species to adapt themselves to induced ischemia without necrosis. Goldblatt et al. (4) find that a high degree of ischemia can be produced in dogs without necrosis. Goldblatt (3) has reported similar findings in the monkey. Any such constriction of the renal artery would lead to necrosis in the rabbit. Pickering and Prinzmetal[1] have had similar results, as regards necrosis of the rabbit kidney, as reported in this paper. Undoubtedly the much richer system of arterial anastomosis in the dog is responsible to some degree for the difference between it and the rabbit.

SUMMARY

A simple method is described for the production of renal insufficiency in rabbits by limiting the blood flow through one kidney and later

[1] Personal communication.

removing the other. Renal insufficiency of any desired degree of severity can be obtained.

The limitation of blood flow should be produced by allowing the artery to grow up to the size of a loop of thread laid around it when the rabbit is small. Attempts to reduce the blood flow by constricting the renal artery in the adult rabbit usually result in necrosis of the kidney.

The induction of renal insufficiency is usually followed by loss of weight and disturbance of the equilibrium apparatus. Hemorrhages into the intestinal wall and into the lumen of the intestine are frequent.

A moderate degree of anemia, as judged from hematocrit readings, comes on very rapidly after reduction in total kidney mass. The total red blood cell volume seems to assume a new and lower level which is maintained for a long time.

Hypertension is present before the right kidney ablation and becomes more severe after this operation.

A certain degree of hypertension can be produced in adult rabbits by moderate restriction of renal blood flow of one kidney. The severity of this is increased by removal of the other kidney.

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IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS*

I. Demonstration of a Complement Fixation Reaction: Relation of Virus-Neutralizing and Complement-Binding Antibodies

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(Received for publication, June 9, 1938)

The immunological study of the Shope papilloma virus (1) has exceptional interest, for the virus causes enduring, proliferative growths that have the character of tumors (2). In the work now to be reported a complement fixation reaction has been used in addition to the neutralization test previously employed (3). Special attention has been paid to the relation of the virus and the complement-binding antigen in extracts of the growths, and to the relation of the virus-neutralizing and complement-binding antibodies in the sera of animals carrying the tumors.

In the present paper the complement fixation test will be described, and the results presented of experiments to determine its specificity. Tests will also be reported that deal with the relation of the complement-binding and virus-neutralizing antibodies, and with the amount of antibody in the sera of cottontail and domestic rabbits bearing the virus-induced papillomas. In two papers that follow, the properties of the complement-binding antigen will be considered, as also its relation to the virus; and some observations will be reported on the yield of virus, and of the complement-binding antigen, from the papillomas of domestic and cottontail rabbits.

Methods

The procedure adopted was essentially an adaptation of that worked out by Bedson and Bland (4). Briefly it consisted in mixing serum,

* Preliminary notes in *Proc. Soc. Exp. Biol. and Med.*, 1937, **35**, 612; 1938, **37**, 657.

703

complement, and tissue extract (antigen) in proper proportion, and allowing these to stand for 2 hours at room temperature to give time for complement fixation to occur. After this period the sensitized cells were added and the tubes incubated at 37°C. for 30 minutes. Readings were then made immediately, and again after the mixtures had stood overnight in the refrigerator.

Serum was obtained by bleeding from an ear vein or by cardiac puncture under ether anesthesia. The blood was allowed to clot in horizontally placed tubes previously coated on one side with sterile paraffin. After the clot had contracted overnight in the refrigerator, the serum was removed and cleared of cells by centrifugation. The sera were stored without added preservatives in the refrigerator at a temperature of about 4°C. Aseptic technique was invariably used in procuring and handling them, but in spite of this a few became contaminated with bacteria, and these were discarded. Immediately prior to use the sera were diluted as required with 0.9 per cent saline and heated at 56° for 30 minutes. In our experience a small percentage of domestic rabbit sera were found to be anticomplementary when tested in the maximum amounts used in these experiments (1:4, occasionally 1:2), and others became anticomplementary after standing for many months in the refrigerator. Only one anticomplementary wild rabbit serum was encountered out of a great number tested. The results with the anticomplementary sera were discarded. A large number of stored sera from domestic and wild rabbits have been used repeatedly over periods up to 15 months, without notable diminution in complement-binding capacity, or the development of anticomplementary properties.

Complement was obtained by bleeding 3 large guinea pigs 5 cc. from the heart on the afternoon before an experiment. The complement titer of the pooled serum was determined accurately just before each experiment, under conditions such as were to be employed in it: 9 tubes were set up with 0.4 cc. of 0.9 per cent saline in each, to render a volume comparable to that of the tests. Then 0.2 cc. of various dilutions of the pooled serum from 1:12 to 1:48 were run in, and finally 0.4 cc. of sensitized cells. The tubes were put into the water bath at 37°C. for 30 minutes and read immediately thereafter. The highest dilution of serum showing complete hemolysis was taken to represent one hemolytic unit of complement. 2 units of complement or slightly more were used in all of the tests reported here, but in other tests as much as 16 units of complement (the maximum amount tested) has been fixed when a potent antigen and antiserum were mixed. The pooled guinea pig sera were usually found to give complete hemolysis at a dilution between 1:20 and 1:40, and the serum was therefore used in the tests at a dilution of 1:10 to 1:20. Rarely the complement titer was found to be less than 1:20. In these cases the material was deemed unsuitable and therefore not used.

Preparation of the Antigens.—The papilloma virus can be readily recovered

from growths preserved for many months in 50 per cent glycerol; and so can the complement-binding antigen, as will appear. For this reason, and because our supply was great, we have used glycerolated tissues almost wholly as a source of antigen, though extracts of papillomas freshly procured and extracts of growths preserved by drying while frozen have also been found effective as complement-binding antigens.

Extracts of the preserved rabbit papillomas and other rabbit tissues were made by grinding the tissues thoroughly with sand in a mortar and suspending them in the required volume of 0.9 per cent saline (dilutions usually 1:10 or 1:20, sometimes 1:5 to 1:320). The crude suspensions were left overnight (occasionally longer) in the ice box. Just before use they were centrifugalized at 3500 R.P.M. for 5 minutes in the International centrifuge with angle head (51°), and the supernatant fluids removed with pipettes. These were again thrown down at 3500 R.P.M. for 15 minutes and the supernatant fluids, free from gross particles and almost water-clear, were carefully removed. Often the extracts were passed through Berkefeld filters in addition. A few papilloma extracts were slightly anticomplementary in the dilutions used, but it was learned early that the anticomplementary effect could be abolished or reduced by further dilution, or by heating at 56°C. for 30 minutes. All antigens were therefore heated immediately before use as routine, occasionally becoming very slightly opalescent in consequence.

The antigens were generally used on the day following their preparation, but occasionally 3 days or longer elapsed prior to their use. It can be stated, however, that a potent extract tested after standing 4 months in the refrigerator was found to have retained its complement-binding capacity.

Small portions of the glycerolated papillomas of a number of individual wild rabbits have been used repeatedly as sources of antigen with consistent results. Occasionally, however, as a given material became almost exhausted, with only a few remnants of tissue remaining, its effectiveness as a complement-binding antigen became somewhat less, as did its demonstrable content of virus.

Hemolytic System.—A 5 per cent suspension of thrice washed sheep cells in 0.9 per cent saline was made for each experiment. Rabbit amboceptor from 2 sources was used; one constantly throughout the early course of the work, and, when it was exhausted, the second for the remainder. Each was titrated at intervals of one week during the period of its use. The hemolytic titer of the first was constant at a dilution of 1:800, of the second at 1:3200. The 5 per cent sheep cell suspension was mixed in equal parts with the amboceptor (diluted to contain 2 hemolytic units in a volume of 0.2 cc.) and incubated at 37°C. for 10 minutes before it was added to the tubes of the test.

Procedure.—Quantities of 0.2 cc. were used in order to conserve materials. This made it expedient to use small test tubes (pyrex, lipped, round bottom, length 9 cm., internal diameter 9 mm.) and standardized pipettes, and to exercise precision in measurement.

The sera to be tested were first diluted with saline as required, and heated at 56°C. for 30 minutes, then 0.2 cc. of each was placed in the bottom of the appropriate tube. 0.2 cc. of complement (titrated immediately beforehand and diluted to contain 2 hemolytic units in 0.2 cc.) was then run in carefully near the bottom of each tube, and next 0.2 cc. of the antigen. Occasionally antigen was run into the tubes first and serum last, but complement was always added second. The tubes were gently shaken and allowed to stand at room temperature for 2 hours. 10 minutes before this time had elapsed the 5 per cent suspension of washed sheep cells was mixed in equal parts with the amboceptor (containing 2 units in 0.2 cc.) and put into the water bath at 37°C. for 10 minutes. 0.4 cc. of the sensitized cells were then added to all tubes and these put into the water bath at 37°C. for 30 minutes. Readings were recorded immediately thereafter, in terms of fixation:

++++ = complete fixation of complement (no hemolysis)
+++ = about 75% "   "   "   (about 25% ")
++ = "   50%   "   "   "   (" 50% ")
+ =   "   25%   "   "   "   (" 75% ")
± =   "   10%   "   "   "   (" 90% ")

The tubes were kept overnight in the refrigerator and read a second time according to the same scale the following morning. Occasionally the two readings varied slightly, and when this happened the second reading was regarded as representing the real end point of the reaction and was thus recorded finally in the protocols.

Tests for non-specific anticomplementary effect of each serum and antigen were set up concurrently in every experiment. Unless specifically stated in the protocols these contained twice the maximum amount of the material used in the experiment. In the absence of anticomplementary effect of either serum or antigen when tested in double amount, any reading of + or more was considered significant.

Sterile glassware and instruments were regularly employed.

Complement Fixation Tests with the Sera of Cottontail Rabbits

It was necessary to learn at the start whether complement fixation would occur if the serum of rabbits bearing the papillomas was mixed with antigens made from the growths. In a first experiment to test the point, the sera of 6 cottontail rabbits bearing naturally occurring papillomas were tested for capacity to bind complement when mixed with virus-containing extracts of glycerolated papillomas from 2 cottontails. The sera of 5 normal cottontails obtained from the same locality in Kansas were tested concurrently.

Experiment 1.—The 5 normal cottontails as well as the 6 bearing naturally occurring papillomas were kept under observation about 4 months before they were bled. At the time of bleeding one of the latter (W.R. 6) had only 3 tiny growths, the largest 1 x 0.4 cm. across and 8 mm. high. Another (W.R. 7) bore a single fleshy papilloma, 2.4 x 2.0 cm. across and 1 cm. high; while each of the 4 remaining animals (W.R. 8, 9, 10, 11) had 7 to 9 large, fleshy, onion-like or discoid papillomas, 2 to 4 cm. across, with peaks 2 to 4 cm. high. The growths were removed from all and put separately into 50 per cent glycerol-Locke's solution. Later the tissues were weighed, after the glycerol had been allowed to drain away and the papillomas dried on sterile gauze.

Two extracts of wild rabbit papillomas were used as antigens. The naturally occurring papillomas of W.R. 1240 had been in 50 per cent glycerol for 33 months. Many previous tests had shown that extracts of these contained virus in high titer. The experimentally induced papillomas of W.R. 42-N had been in glycerol for 16 months. Extracts of these papillomas were known to be infectious also, but much less so than were those of W.R. 1240. 1 gm. of the glycerolated tissue from each animal was ground separately and made up to a volume of 20 cc. with 0.9 per cent saline. These crude suspensions were left overnight in the refrigerator, then centrifugalized twice at 3500 R.P.M. for 5 and 15 minutes, with removal of the second, water-clear supernatant fluids for use. The tests were set up in the routine way previously described.

The findings set down in Table I make it evident that complement fixation does occur when saline extracts of the virus-induced papillomas are mixed with the sera of rabbits bearing the growths: all of the sera of the 6 cottontail rabbits bearing naturally occurring papillomas bound complement in the test, while the sera of the 5 normal cottontails did not do so. It will be noted further that the serum of W.R. 6, a rabbit with 3 tiny papillomas weighing only 300 mg. all told, fixed complement hardly at all; and the serum of W.R. 7, an animal bearing a single, good sized, fleshy growth, bound it only partly. The remaining 4 sera, from cottontails with several large, fleshy growths, gave practically complete fixation. These findings suggest a certain relation between the amount of papilloma tissue borne by an animal and the capacity of its serum to bind complement, a point which will receive further consideration later on.

In a second experiment the sera of 5 cottontails, which had borne experimentally induced papillomas for a considerable time, were similarly tested for complement-binding capacity. The sera of only 3 normal cottontails were available for comparison.

Experiment 2.—The 5 cottontails had been inoculated with 2 potent strains of virus into small areas of shaved skin on each flank with the tattoo machine. Growths appeared in all at every inoculated site after 15 to 25 days. In only one animal were the growths notably vigorous, but all were kept until 136 days had elapsed. When bled on this day one animal (W.R. 16) had 3 fleshy growths 2 to 3 cm. across and 1 cm. high; whereas complete retrogression of 2 growths had

TABLE I

Complement Fixation Tests with the Sera of Normal Cottontail Rabbits and of Others with Naturally Occurring Papillomas

Source of serum	Cottontail rabbit No.	Weight of the papillomas carried by the rabbit	Complement fixation tests		
			Antigen W.R. 1240	Antigen W.R. 42-N	Serum controls (no antigen)
Normal cottontail rabbits	W.R. 1	gm.			
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
Cottontail rabbits bearing naturally occurring papillomas		0	0	0	0
	6	0.3			0
	7	3.2	±	++	0
	8	10.5	++	+++	0
	9	14.4	++++	++++±	0
	10	17.4	+++±	+++	0
	11	22.9	++++	+++±	0
Antigen controls (no serum).................			++++	+++	0
			0	0	0

Complement, 2 units in all tubes.
Sera diluted 1:4 in the tests with antigens, 1:2 in control tests.
Antigens, 1:20 saline extracts of glycerolated papillomas from 2 cotton··· rabbits.

++++ = complete fixation (no hemolysis).
+++ = about 75% "
++ = " 50% "
+ = " 25% "
± = " 10% "
0 = no fixation (complete hemolysis).
W.R. = wild rabbit.
D.R. = domestic rabbit.

occurred in another (W.R. 17). The remaining 3 each bore one or two small, dry papillomas.

None of the 3 normal cottontail sera fixed complement in this test (Table II), while the sera of all of the 5 rabbits bearing the experimentally induced growths bound it completely.

In a third experiment the sera of 6 other cottontail rabbits were tested under broader conditions, to see if a relation exists between the amount of papilloma tissue borne by an animal and the complement-binding capacity of its serum.

TABLE II

Complement Fixation Tests with the Sera of Normal Cottontail Rabbits and of Others Bearing Experimentally Induced Papillomas

Source of serum	Cottontail rabbit No.	Complement fixation tests	
		Antigen W.R. 33-N	Serum controls (no antigen)
Normal cottontail rabbits	W.R. 12	0	0
	13	0	0
	14	0	0
Cottontail rabbits bearing experimentally induced papillomas	15	++++	0
	16	++++	0
	17	++++	0
	18	++++	0
	19	++++	0
Antigen control (no serum)..................		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 saline extract of glycerolated cottontail papillomas.

Experiment 3.—To produce papilloma material for various purposes, a 10 per cent extract of the glycerolated growths of W.R. 7-N was rubbed into scarified areas on both sides of 20 normal cottontail rabbits. After 14 days confluent and semiconfluent growths had appeared in many of the animals, and soon thereafter in the remainder. A number of the rabbits were killed early, to procure their papillomas, and their sera were not taken. 8 individuals were kept, however, and these were bled from the heart at various times between the 28th and 116th days, records being made from time to time of the size and character of their growths. The sera were stored in the refrigerator for 4 months, during which time 2 specimens became cloudy owing to growth of bacteria, and hence were discarded. The 6 remaining sera were tested according to the routine procedure in dilutions of 1:4, 1:8, 1:16, and 1:32, with a 1:20 extract of W.R. 1240 papillomas as antigen.

TABLE III

Complement Fixation Tests with the Sera of Cottontail Rabbits Bearing Experimentally Induced Papillomas

Cottontail rabbit No.	Character and size of the growths when serum was procured	Day after virus inoculation when serum was procured	Complement fixation tests				Serum controls (no antigen)
			Serum dilutions				
			1:4	1:8	1:16	1:32	
W.R. 20	A few tiny, scattered, dry, discrete papillomas on both sides, none over 1 mm. high	28th	0	0	0	0	0
21	A few discrete, semiconfluent, dry papillomas on both sides, 2 mm. high. All had retrogressed by the 83rd day	83rd	0	0	0	0	0
22	9–10 discrete and semiconfluent growths on both sides, 1 cm. high	83rd	++++	++++	0	0	0
23	Semiconfluent growths over areas 6 x 4 cm. on both sides 1 cm. high	69th	++++	++++	++++	±	0
24	Confluent, fleshy papillomatous masses over areas 6 x 4 cm. on both sides, with peaks 3 cm. high	69th	++++	++++	++++	+++±	0
25	Confluent, fleshy papillomatous masses over areas 6 x 4 cm. on both sides with peaks 3½ cm. high	113th	++++	++++	++++	++++	0
Antigen control (no serum).............			0				

Complement, 2 units in all tubes.

Antigen, 1:20 saline extract of glycerolated papillomas (W.R. 1240).

Two of the sera (from W.R. 20 and 21) did not fix complement under the conditions of the test (Table III). These rabbits had borne only a few small growths over comparatively short periods. (W.R. 21 had been bled on the 83rd day, but its small growths had retrogressed completely several weeks previously.)

The serum of W.R. 22, withdrawn on the 83rd day, fixed complement completely in a dilution of 1:8, but not at all in a dilution of 1:16; while the serum of W.R. 24, secured a fortnight previously, gave complete or almost complete fixation in all dilutions up to 1:32. A reason for the difference in serum-antibody titer is at once apparent in the different course of the growths in these 2 animals, the papillomas of W.R. 24 being much more numerous and much larger on the 68th day than were those of W.R. 22 on the 83rd day. W.R. 25 had borne large, confluent growths for a long period, and its serum fixed complement in all dilutions tested.

The findings in this experiment (Table III) confirm and extend those already presented. It is evident that the sera of cottontail rabbits bearing the virus-induced papillomas, whether naturally occurring or experimentally induced, will fix complement in the presence of extracts of the growths, while the sera of normal cottontails have exhibited no such capacity. There is, furthermore, an evident relation between the total mass of papilloma tissue borne by an animal and the complement-binding capacity of its serum: in general, the sera of rabbits that have borne large growths over long periods fix complement in higher titer than the sera of others with small growths of shorter duration.

Tests with the Sera of Domestic Rabbits

The Shope virus causes characteristic papillomas when inoculated experimentally into the skin of domestic rabbits, and most of these animals develop virus-neutralizing antibodies after carrying the growths for a while (1, 3). Will their sera fix complement in the presence of suitable antigens, as the sera of cottontails with virus-induced papillomas have just been shown to do? To test the point, the sera were tested of 5 normal domestic rabbits and of 5 others that had borne large virus-induced papillomas for a considerable period.

Experiment 4.—5 normal domestic rabbits (Belted Dutch) had been inoculated with a 5 per cent suspension of W.R. 1240 virus by rubbing it into large scarified areas (about 9 x 10 cm.) on each flank. 8 days later small papillomas had appeared, scattered rather thickly over every inoculated site. These rapidly enlarged into large, confluent papillomatous expanses in every animal; but in 2 of them (D.R. 5 and 6) the growths were somewhat larger, fleshier, and more vigorous than in the remainder. By the 52nd day all bore huge, confluent papillomatous masses on both sides, 2 to 3 cm. high. All were bled 50 cc. from the

heart on this day, as were 5 normal Dutch rabbits from the same breeding colony, which has been kept in an isolated room for control purposes. After storage in the ice box for approximately 5 weeks the sera were tested for ability to bind complement in the presence of an antigen consisting of a freshly prepared 10 per cent Berkefeld V filtrate of the glycerolated papillomas of W.R. Tx, known from previous tests to contain virus in considerable amount.

TABLE IV

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Others Bearing Large, Experimentally Induced Papillomas

Source of serum	Domestic rabbit No.	Character and size of growths on both sides	Complement fixation tests			Serum controls (no antigen)
			Serum dilutions			
			1:2	1:4	1:6	
Normal domestic rabbits	1	Nil, control				
	2	" "	0	0	0	0
	3	" "	0	0	0	0
	4	" "	0	0	0	0
	5	" "	0	0	0	0
			0	0	0	0
Domestic rabbits bearing large, experimentally induced papillomas	6	Confluent, 12 x 11 cm.				0
	7	" 12 x 10 "	++++	++++	++++	0
	8	" 9 x 9 "	++++	++++	++++	0
	9	" 10 x 8 "	++++	++++	++++	0
	10	" 10 x 10 "	++++	+++±	+++	0
Antigen control (no serum).................			+++±	++±	++	0
			0			

Complement, 2 units in all tubes.
Sera diluted as indicated. All were procured on the 52nd day after inoculation.
Antigen, 1:10 Berkefeld V filtrate of cottontail papillomas (W.R. Tx).

None of the normal sera fixed complement, while the sera from the 5 papillomatous rabbits gave fixation in the 3 dilutions tested (Table IV).

In the following experiment the findings were extended by testing the sera of domestic rabbits that had borne somewhat smaller growths but over a longer period of time. 2 normal sera were included for comparison.

Experiment 5.—10 Dutch rabbits that had carried papillomas for long periods were bled. 2 of these (D.R. 11 and 12) had borne for 11 months 18 discrete

papillomas, the outcome of tattoo inoculation of virus. When the serum was procured the growths were large and discoid, averaging 3.5 cm. in diameter. 3 other animals (D.R. 13, 14, and 15) had been inoculated 4 months previously by tattooing virus into 6 small areas (2 mm. in diameter) on each side. When bled, these carried 12 discoid papillomas 2 to 4 cm. across. The remaining 5 rabbits bore 4, 5, or 6 discrete, semiconfluent or confluent growths on each side, which had been brought about by rubbing 4, 5, or 6 strains of virus into as many scarified rectangles on each side 6 months previously.

TABLE V

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Others Bearing Experimentally Induced Papillomas

Source of serum	Domestic rabbit No.	Antigen W.R. 33-N	Serum controls (no antigen)
Normal domestic rabbits	1	0	0
	2	0	0
Domestic rabbits bearing large experimentally induced papillomas	11	++++	0
	12	++++	0
	13	++++	0
	14	++++	0
	15	++++	0
	16	++++	0
	17	++++	0
	18	++±	0
	19	++	0
	20	+	0
Antigen control (no serum).................		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in the tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

The sera were tested according to the routine procedure, with an antigen consisting of a 10 per cent extract of the glycerolated infectious papillomas of W.R. 33-N prepared as usual on the preceding day.

The results of the experiment are summarized in Table V. 7 of the 10 sera gave complete fixation, one (D.R. 20) gave almost none, while 2 others (D.R. 18 and 19) showed only moderate capacity to bind complement. The results with these latter 3 sera evidently represented variations in host reactions, for the papillomas of these

rabbits were as large and numerous as in some of the others, and they had been present for 6 months.

Experiments 4 and 5 show that the sera of domestic rabbits bearing experimentally induced papillomas generally possess the capacity to bind complement when mixed with antigens containing the virus, while the sera of normal domestic rabbits have no such ability.

Tests with Sera from Animals Carrying Tar Papillomas

It is conceivable that the results of the foregoing tests depend upon some constituent present in papillomas generally. For this reason an experiment was done next to see whether or not the sera of rabbits bearing papillomas elicited by tarring would bind complement under similar conditions.

Experiment 6.—Sera were procured from 6 gray-brown domestic rabbits bearing papillomas elicited by repeated applications of tar to the inner surfaces of their ears. All of these animals had been kept in isolation. One (D.R. 21) had been tarred twice weekly for 11 months. It bore 19 tar papillomas up to 7 mm. across and 1 cm. high. Another (D.R. 22) had been tarred similarly for 5 months: it had 4 large growths, one 2 cm. in diameter. The remainder had been tarred for 3½ months: all had 8 to 12 tar papillomas up to 1 cm. in diameter. The sera were tested as usual with an antigen consisting of a Berkefeld V filtrate of the glycerolated papillomas of W.R. Tx.

In this test (Table VI) none of the sera from the rabbits bearing tar papillomas fixed complement in the presence of a potent antigen made from the virus-induced papillomas, whereas specimens from 2 domestic rabbits with growths of the latter sort bound it completely.

Tests with Sera from Animals Immune to the Shope Fibroma Virus

To test the specificity of the reaction further, sera were now employed from domestic rabbits immune to the fibroma virus as result of previous infection with it, and from others immune both to the fibroma virus and to myxoma virus.

Experiment 7.—The sera were generously provided by Dr. Shope. 2 were from domestic rabbits immune to the inflammatory strain of the fibroma virus. 7 others were from domestic rabbits in which fibromas had retrogressed and in which test inoculations with the myxoma virus had proven negative. All of the sera had been kept from 15 to 27 months in the refrigerator yet all were clear. They were tested in the routine way with an antigen consisting of a

freshly prepared 10 per cent Berkefeld V filtrate of the infectious papilloma of W.R. Tx.

In this experiment (Table VII) none of the sera from 2 rabbits immune to the fibroma virus and from 7 rabbits immune to the fibroma and myxoma viruses fixed complement in the presence of a potent papilloma antigen. The results are open to some question, however, because the sera had been kept for a long period before testing. In this connection it may be pointed out, though, that we have recently

TABLE VI

Complement Fixation Tests with the Sera of Domestic Rabbits Bearing Virus-Induced Papillomas and of Others with Papillomas Elicited by Tarring

Source of serum	Domestic rabbit No.	Antigen W.R. Tx	Serum controls (no antigen)
Domestic rabbits bearing papillomas elicited by tarring	21	0	0
	22	0	0
	23	0	0
	24	0	0
	25	0	0
	26	0	0
Domestic rabbits bearing virus-induced papillomas	7	++++	0
	8	++++	0
Antigen control (no serum)...............		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

tested 3 sera that had been kept for 12 to 15 months in the refrigerator. None had become anticomplementary and all had retained their capacity to bind complement without perceptible loss in titer.

Further Control Tests on Specificity

In further elucidation of the specificity of the complement fixation reaction a test was done with the sera of 3 rabbits immune to vaccine virus, 4 with experimental syphilis, and one hyperimmunized against herpes virus.

Experiment 8.—The serum of the domestic rabbit hyperimmunized against herpes virus and of the 3 rabbits immune to vaccinia were generously provided by Drs. Rivers and Smadel. Dr. T. B. Turner kindly sent sera from 4 rabbits with experimental syphilis. The serum of one of these latter (D.R. 40) had been found markedly positive when tested according to the routine Kolmer-Wassermann technique. The others had not been tested. None had been kept longer than 4 months. In the present experiment, sera from 2 domestic rabbits with experimental papillomas were used for comparison: these had been kept 5 months

TABLE VII

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Those Immune to the Fibroma and Myxoma Viruses

Source of serum	Domestic rabbit No.	Antigen W.R. Tx	Serum controls (no antigen)
Domestic rabbits immune to fibroma virus	27	0	0
	28	0	0
Domestic rabbits immune to both fibroma and myxoma viruses	29	0	0
	30	0	0
	31	0	0
	32	0	0
	33	0	0
	34	0	0
	35	0	0
Domestic rabbits bearing virus-induced papillomas	7	++++	0
	8	++++	0
Antigen control (no serum)		0	0

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

in the refrigerator. All the specimens were tested in a dilution of 1:2 with 2 potent antigens. One antigen consisted of a 1:40 suspension of the glycerolated papillomas of W.R. 8-N, a notably infectious material; while the second was made by extracting the infectious papillomas of W.R. 33-N in a dilution of 1:10.

In this experiment (Table VIII) none of the sera from domestic rabbits immune to vaccinia or herpes viruses, or with experimental syphilis, fixed complement specifically with either of the 2 antigens, while both of the sera from papilloma-bearing rabbits bound it com-

letely. It will be noted that there was a slight amount of fixation by 3 of the sera (D.R. 38, 41, and 43) in the control tests as well as with the antigens. This amount of fixation was deemed insignificant, being attributable to non-specific anticomplementary effect, perhaps consequent on the fact that the sera were used in a dilution of 1:2 and had stood for some months before being utilized.

TABLE VIII

Complement Fixation Tests with the Sera of Domestic Rabbits Immune to Vaccine Virus, and of Others with Experimental Syphilis

Source of serum	Domestic rabbit No.	Antigens		Serum controls (no antigen)
		W.R. 8-N 1:40	W.R. 33-N 1:10	
Rabbits immune to vaccine virus	36	0	0	0
	37	0	0	0
	38	0	±	±
Rabbit hyperimmunized against herpes virus	39	0	0	0
Rabbits with experimental syphilis	40	0	0	0
	41	±	±	±
	42	0	0	0
	43	+	+	±
Rabbits bearing virus-induced papillomas	6	++++	++++	0
	7	++++	++++	0
Antigen controls (no serum).............		0	0	

Complement, 2 units in all tubes.
All sera diluted 1:2.
Antigens, saline extracts of glycerolated cottontail papillomas from 2 rabbits.

The findings indicate that the complement fixation test has a specificity comparable to that of the neutralization test involving the same virus, for it is known that sera from rabbits bearing tar papillomas, or from rabbits immune to the fibroma, myxoma, or vaccine viruses, do not neutralize the papilloma virus (3). Manifestly the humoral principles responsible for complement fixation are engendered by a specific antigen liberated from the virus-induced papillomas.

Comparative Titers of the Sera of Cottontail and Domestic Rabbits

The sera of the cottontail rabbits thus far tested (Tables I, II, and III) fixed complement in higher titer than those of domestic rabbits bearing comparable growths (Tables IV and V). In order to learn whether this relation holds true generally, a number of sera were tested from rabbits of both species bearing virus-induced papillomas of different size and duration.

Experiment 9.—5 belted-Dutch domestic rabbits, each bearing 8 large papillomas, were bled 5 cc. from an ear vein. These had been selected at random from a group of 26 related rabbits, all of which had been inoculated 100 days before by rubbing 4 potent strains of virus into as many scarified rectangles on each side. All of the growths on the selected rabbits were large, fleshy and vigorous, averaging 3.5 x 5 cm. in area, with keratinized peaks rising 2 to 3 cm. above the skin level; and there was little difference from rabbit to rabbit, the papillomas of D.R. 48 being only slightly larger and fleshier than those of its fellows. 3 sera previously used from cottontails with large papillomas were again tested, and 2 others. These latter had been recently procured from 2 animals inoculated 86 days before by rubbing a potent 10 per cent virus fluid (W.R. 7-N) into scarified areas about 6 x 8 cm. on their abdomens. The growths of W.R. 27 had been confluent and very vigorous from the start, with a number of rounded subcutaneous pearls beneath them; while those of its fellow (W.R. 26) had always been discrete and much smaller.

All of the sera were tested in various dilutions with 2 antigens consisting of freshly prepared 10 per cent Berkefeld V filtrates of glycerolated materials from two sources, both containing virus in high titer.

The findings are summarized in Table IX, from which it will be seen that the sera of only one of the domestic rabbits (D.R. 48) bound complement in significant titer, whereas 4 of the 5 cottontail sera bound it in much higher titer. The serum of W.R. 26, the cottontail with comparatively small growths, gave practically no fixation with either antigen. Before these findings are appraised it should be pointed out again that both antigens consisted of 1:10 filtrates of notably pathogenic materials with great complement-binding capacity. Some of the results may have been due to inhibition of fixation in the presence of excess of antigen (prozone phenomenon[1]).

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[1] This phenomenon has been encountered a number of times throughout the work, with inhibition of fixation when there was a large excess of either antigen or antibody; and in an experiment which need not be given in detail the phenomenon was purposely demonstrated. Precipitation reactions have also been observed a number of times in collateral tests, when potent antigens and antisera were mixed and incubated at 37°C. for several hours.

TABLE IX

Complement Fixation Tests with the Sera of Domestic and Cottontail Rabbits Bearing Virus-Induced Papillomas

Source of serum	Rabbit No.	Complement fixation tests										Serum controls (no antigen)
		Antigen W.R. 35					Antigen W.R. 1240					
		Dilutions of sera					Dilutions of sera					
		1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	
Domestic rabbits bearing virus-induced papillomas	44	0	0	0	0	0	0	0	0	0	0	0
	45	0	0	0	0	0	±	0	0	0	0	0
	46	0	0	0	0	0	0	0	0	0	0	0
	47	+±	+	+±	0	0	++	++	0	0	0	0
	48	++++	++++	+++	+++	0	++	+±	0	0	0	0
Cottontail rabbits bearing virus-induced papillomas	26	±	0	0	0	0	0	0	0	0	0	0
	22	++++	++++	++	++	0	++	++	0	0	0	0
	24	+++++	++++	+++	+++	±	++++	++++	+++	++	+	0
	25	+++++	++++	+++	++	0	++++	++++	+++	+++	+	0
	27	+++++	+++++	++++	++++	++	++++	++++	++++	+++	+±	0
Antigen controls (no serum)......		0					0					

Complement, 2 units in all tubes.
Antigens, 1:10 Berkefeld V filtrates of glycerolated papillomas.

The cottontail serum that failed to bind complement in significant titer came from a rabbit bearing relatively small growths, as compared with its fellows; but no such obvious difference exists to account for the very wide variations in the titer of the domestic rabbit sera. The growths in these animals were produced with the same inoculum, and they differed but little from animal to animal,—all being large, fleshy, and vigorous, and of the same duration. Manifestly individual differences played a large part in the results, as in the case of virus neutralization (1, 3).

In this experiment the sera of cottontail rabbits bearing large papillomas bound complement in much higher titer than the sera of domestic rabbits with comparable growths. Many subsequent tests have shown this to be a general rule.

Relation of the Complement-Binding and Virus-Neutralizing Antibodies

The foregoing experiments have shown that the sera of cottontail and domestic rabbits bearing virus-induced papillomas will bind complement when mixed with antigens consisting of extracts of the growths. From previous work (3) it was known that the sera of rabbits with papillomas will generally neutralize the virus when mixed with it *in vitro*. What relation do these humoral principles bear to one another? Do the complement-binding and virus-neutralizing antibodies exist in the same relative proportions in the blood?

In the first of two experiments to decide this question 6 domestic rabbit sera, known to differ in their capacity to bind complement, were tested for ability to neutralize the virus.

Experiment 10.—The complement-binding titers were determined by testing the sera in dilutions of 1:2, 1:4, 1:6, 1:8, and 1:10, with an antigen consisting of a freshly prepared 5 per cent suspension of W.R. 33-N papillomas. On the same day neutralization tests were made with the same serum specimens by incubating mixtures of the serum in equal parts with a virus filtrate (containing approximately 400 infective units of virus per inoculation dose) and inoculating these into scarified rectangles on the skin of 3 normal domestic rabbits, according to a standard procedure (3). The mixtures were incubated for 2 hours at 37°C. before inoculation. The lesions on the test animals were recorded at intervals of 3 to 4 days between the 14th and 33rd days. In order to conserve space the readings on but one test rabbit, on the 29th day, are given in the table. These express accurately the differences in neutralizing capacity of the sera as manifested by the entire course of the lesions on all 3 test animals.

It will be seen (Table X) that the serum of D.R. 6 bound complement com-

pletely in dilutions of 1:2 and 1:4, and almost completely when diluted 1:8 and 1:10; while the serum of D.R. 8 bound it almost as well. These 2 sera neutralized the 5 per cent virus filtrate (about 2000 minimal infective doses) completely, and did so almost completely when diluted 1:2. The sera of D.R. 18 and 19 fixed the complement only partially in dilutions of 1:2 and 1:4; and these neutralized the 5 per cent virus only partially, although neutralizing 1 per cent virus com-

TABLE X

Neutralization and Complement Fixation Tests with the Sera of Six Domestic Rabbits Bearing Virus-Induced Papillomas

Serum	Neutralization tests*			Complement fixation tests					
Domestic rabbit No.	Whole serum + 1 per cent virus	Whole serum + 5 per cent virus	Serum 1:2 + 5 per cent virus	Dilutions of sera					Serum controls (no antigen)
				1:2	1:4	1:6	1:8	1:10	
6	++++	++++	+++±	++++	++++	+++	+++	+++	0
8	++++	++++	+++	++++	++++	+++	++	+	0
18	++++	++±	++	+++	++	+	0	0	0
19	++++	++	++	++	±	0	0	0	0
44	+++	++	n.t.	+	0	0	0	0	0
20	+++	±	n.t.	±	0	0	0	0	0
Antigen control (no serum).......			0						

Complement, 2 units in all tubes.
Antigen, 1:20 saline extract of glycerolated papillomas (W.R. 33-N).
   * ++++ = complete neutralization (no growth at the inoculation site).
     +++ = about 75% neutralization.
     ++ =   "   50%   "
     + =   "   25%   "
     ± =   "   10%   "
     0 = no neutralization (*i.e.*, confluent growths as from the virus-saline control mixtures).
     n.t. = not tested.

pletely. The sera of D.R. 44 and 20 showed little capacity to bind complement, even in dilutions of 1:2; but they neutralized the 1 per cent virus almost completely, and a 0.2 per cent dilution of the virus completely, as was shown in other tests not recorded in the table.

It is evident that the complement fixation titers of these sera varied in parallel with their virus neutralization titers. Furthermore it is plain that sera can possess a considerable virus-neutralizing capacity

without being able to bind complement to a noticeable degree, at least under the circumstances of the test. The findings pose the question whether the neutralization test is more sensitive than the complement fixation reaction, a matter that will be taken up after the next experiment.

In order to extend the findings use was now made of a fact learned in Experiment 9,—namely that the sera of many cottontail rabbits with virus-induced papillomas will bind complement in much higher titer than sera from domestic rabbits with comparable growths. Acting on this knowledge, domestic and cottontail sera known to vary widely in complement fixation titers were compared as to content of virus-neutralizing and complement-binding antibodies.

Experiment 11.—The serum of D.R. 46, which had not bound complement in the test of Experiment 10, and that of D.R. 48, which had bound it completely at dilutions of 1:4 and 1:8, and partially at 1:16, were selected for the neutralization tests; as were also the sera of W.R. 26 and 27, the former having shown practically no capacity to bind complement (Experiment 9), whereas the latter fixed it completely in all dilutions up to 1:32, and partially at 1:64. A normal domestic rabbit serum (D.R. 49) and a normal cottontail serum (W.R. 2) were included for comparison. Neither of these had bound complement in many tests with various potent antigens.

The neutralization tests were done in the standard way (3), with several dilutions of the sera mixed in equal parts with a centrifugalized, almost water-clear, 5 per cent suspension of glycerolated papillomas from cottontail 56, this material being slightly more potent than a 5 per cent filtrate of W.R. 1240 virus. The mixtures were put into the water bath for 2 hours at 37°C. before they were rubbed into scarified skin areas on the 3 test animals.

Table XI shows the results of the experiment. The neutralization readings are those with one test animal on the 20th day, according to the scale of Experiment 10. The 2 normal sera failed to bind complement, and they did not neutralize the virus. Mixtures of these sera in equal parts with the 5 per cent virus fluid produced confluent areas of papillomatosis covering the entire inoculation site, and wholly similar to those produced by the virus-saline control mixtures. The serum of D.R. 46, which did not fix complement at all, neutralized the virus partially when undiluted, but had no great effect on it when diluted 1:4 or 1:16. The serum of W.R. 26 neutralized the virus (more than 2000 infective doses) almost completely when undiluted, and partially when diluted 1:4, but only slightly at 1:16. This serum had bound complement slightly. The serum of D.R. 48, and that of W.R. 27, neutralized the virus in high titer, and bound complement likewise.

Nine of the 10 sera of these two experiments had parallel complement fixation and neutralization titers, the range of which varied greatly. One of them neutralized the virus in low titer, yet failed to bind complement in the tests; and several neutralized the virus in greater or less amount, but fixed complement only slightly or not at all. It is plain, therefore, that the complement-binding and virus-neutralizing antibodies exist in the same relative proportions in the

TABLE XI

Neutralization Tests with Cottontail and Domestic Rabbit Sera with Markedly Different Complement Fixation Titers

Serum	Neutralization tests			Complement fixation tests				
	5 per cent virus (W.R. 56) +			Dilutions of sera				
Rabbit No.	Whole serum	Serum 1:4	Serum 1:16	1:4	1:8	1:16	1:32	1:64
D.R. 49	0	0	0	0	0	0	0	0
46	++	+	0	0	0	0	0	0
48	++++	+++	+±	++++	++++	+±	0	0
W.R. 2	0	0	0	0	0	0	0	0
26	+++±	+	±	±	0	0	0	0
27	++++	++++	+++±	++++	++++	++++	++++	+++

+++ = complete neutralization or complete fixation.
Complement, 2 units in all tubes.
Antigen, 1:10 Berkefeld V filtrate of glycerolated papillomas of W.R. 35.
Control tests of antigen and of all sera in double amount showed no anti-complementary effect.

sera of rabbits bearing the papillomas; but the neutralization test appears to have a "lower threshold" than the complement fixation reaction, although it is not notably more sensitive in the effective range of the latter.

SUMMARY

The sera of rabbits bearing virus-induced papillomas have been found to bind complement when mixed with antigens consisting of extracts or filtrates of the growths containing the virus. The sera of normal rabbits, of those immune to other viruses (vaccinia, herpes, fibroma, myxoma), of rabbits with syphilis, or of those with papillomas

consequent on tarring, did not fix complement upon admixture with the papilloma antigens.

The complement-binding antibody was present in the serum specimens in the same relative proportions as the virus-neutralizing antibody, and both were present in greatest amount in the sera of rabbits that had borne large papillomas over considerable periods of time.   A few sera were come upon that neutralized small amounts of the virus yet failed to bind complement to any noteworthy degree in the tests.

The sera of cottontail rabbits fixed complement and neutralized the virus in much higher titer than the sera of domestic rabbits with comparable growths.

The implications of the findings will be discussed in a subsequent paper, after the properties of the complement-binding antigen have been scrutinized.

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IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS

II. Properties of the Complement-Binding Antigen Present in Extracts of the Growths: Its Relation to the Virus

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The sera of rabbits bearing virus-induced papillomas will fix complement in the presence of antigens consisting of saline extracts of the growths, as the experiments of Paper I have shown. The nature of the complement-binding antigen and its relation to the papilloma virus will now be considered.

Tests with Extracts of Normal Skin

Since the papillomas consist of epidermal cells infected with the virus it was necessary to learn first whether the antigen exists only in papillomatous tissue or whether it can be extracted from normal epidermal tissue also. In an experiment to decide the point, portions of the skin and of the naturally occurring papillomas of 3 cottontail rabbits were extracted and tested for capacity to bind complement in the presence of known immune sera, according to the technique described in Paper I.

Experiment 12.—Using aseptic technique, a disk of skin weighing approximately 500 mg. was removed from the flank of each of the 3 cottontails, and similar amounts of the naturally occurring papillomas of the same animals were also procured. The materials were weighed and ground separately, and saline added to them to make 1:20 suspensions. They were left overnight in the refrigerator and then centrifugalized twice, first at 3500 R.P.M. for 5 minutes, after which the supernatant fluids were again spun at 3500 R.P.M. for 10 minutes. The water-clear extracts were then heated at 56°C. for 30 minutes as usual and tested for capacity to bind complement. Sera were used from 4 cottontails bearing experimentally induced growths, 2 of which had been "hyperimmunized" by means of

repeated injections of Berkefeld filtrates of the growths. One of the hyperimmune sera (W.R. 8-S) had a complement fixation titer of 1:128 when tested with potent antigens, the other 1:64. The sera of 2 normal cottontails were included for comparison.

In this experiment (Table XII) none of the specific complement-binding antigen could be extracted from the normal skin of cottontail rabbits though it was obtained in large amount from the virus-induced papillomas of the same animals. The objection might be raised that the skin of domestic rabbits is a tissue consisting mostly of dermis, with

TABLE XII

Complement Fixation Tests with Extracts of Normal Skin and of the Naturally Occurring Papillomas of Three Cottontail Rabbits

W.R. No.	Antigens Tissue	Immune		Hyperimmune		Normal		Antigen controls (no serum)
		15-S	19-S	8-S	12-S	30-T	36-T	
53	Skin	0	0	0	0	0	0	0
56	"	0	0	0	0	0	0	0
55	"	0	0	0	0	0	0	0
53	Papilloma	++++	++++	++++	++++	0	0	0
56	"	++++	++++	++++	++++	0	0	0
55	"	++	+++	++++	++±	0	0	0
Serum controls (no antigen)..........		0	0	0	0	0	0	0

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigens, 1:20 extracts of fresh tissues.

a relatively small proportion of epidermal cells, a material notably difficult to extract. But these objections do not hold in the case of cottontails, for their skin is quite thin, consisting almost wholly of epidermis with but a shallow web-like corium underneath, and it can be readily ground in mortar.

Filtration of the Complement-Binding Antigen

An experiment was next undertaken to find whether the antigen is held back by filters that retain the virus. The latter is known to

pass readily through Berkefeld V filters, but in our experience, most of it has been retained on filtration through Berkefeld W candles or Seitz pads.

Experiment 13.—5 per cent suspensions of the glycerolated, infectious papillomas of W.R. 8-N and W.R. Tx were made as usual, and allowed to stand 7 and 20 days respectively in the refrigerator, to give time for the dissociation of any

TABLE XIII

Pathogenicity and Complement Fixation Tests with Berkefeld V and W and Seitz Filtrates of Cottontail Papillomas

Papilloma extracts		Pathogenicity tests*			Complement fixation tests		
		Test rabbits			Dilution of extracts		
Source	Preparation	A	B	C	1:20	1:100	1:500
W.R. 8-N	Unfiltered	++++	++++	++++	++†	++++	0
	V filtrate	+++±	++++	++++	++++	++	0
	W "	±	+	+	±	0	0
	S "	+	±	+±	0	0	0
W.R. Tx	Unfiltered	+++	+++	+++	++++	+	0
	V filtrate	+++	++±	+±	++	0	0
	W "	+	+	+	±	0	0
	S "	0	±	0	0	0	0

Complement, 2 units in all tubes.

Immune serum D.R. 7 diluted 1:4.

Papilloma extracts diluted 1:100 in pathogenicity tests,—as indicated in complement fixation tests.

The immune serum and papilloma extracts showed no anticomplementary effect when tested in double amount in control tests.

* Readings made on the 42nd day after inoculation, according to the standard scale.

++++ = confluent papillomatosis.
+++ = semiconfluent "
++   = many discrete papillomas.
+   = a few papillomas.
±   = 2, 3, or 4 discrete papillomas.
±   = 1 papilloma.
† Prozone effect.

soluble constituents (1). The extracts were then centrifugalized twice as usual and the supernatant fluids removed. 5 cc. aliquots of these were then filtered through Berkefeld V and W filters (size 5) under negative pressure of about 30 cm.

of water, and through single Seitz EK disks under a positive pressure of 5 pounds. The filtrates were water-clear. About 4 cc. was obtained by filtration through the Seitz pads, while only 1.5 to 2.0 cc. came through the Berkefeld candles. The unfiltered extracts as well as the filtrates were tested for pathogenicity according to the routine method (2), and for complement-binding capacity in the presence of a known immune serum. Table XIII shows the findings. Both of the unfiltered extracts were highly pathogenic, and they bound complement completely, that of W.R. 8-N being the more active in both respects. The Berkefeld V filtrates of both materials showed a slight loss in pathogenicity and complement fixation titer, while the Berkefeld W and Seitz filtrates showed a marked or complete loss of both.

The findings (Table XIII) show clearly that the complement-binding antigen was retained by the filters in almost precisely the same proportions as the virus. There was no indication that a "soluble antigen" existed more readily filterable than the virus, nor that aggregates too large to pass through Berkefeld V filters played any significant part in the findings.

Centrifugation of the Complement-Binding Antigen

In the next experiment the findings were extended by a test with the centrifuge, a papilloma extract known to contain much active virus being centrifugalized at widely various speeds, and the supernatant fluids and sediments tested thereafter both for pathogenicity and complement-binding capacity.

Experiment 14.—A 5 per cent extract of the glycerolated, naturally occurring papillomas of W.R. 54 was prepared as usual and let stand overnight in the refrigerator. It was then mixed and spun in the horizontal centrifuge at about 940 R.P.M. for 5 minutes. This amount of centrifugation sufficed to throw down the sand, but little more, and the supernatant fluid was quite turbid. A portion of this was set aside for testing, and 12 cc. of the remainder was centrifugalized in the angle head centrifuge at 4000 R.P.M. for 20 minutes in a round-bottom tube with an internal diameter of 12 mm. and an overall length of 13 cm. This procedure rendered the supernatant fluids almost water-clear, and packed a considerable amount of fine sediment in the bottom of the tube. The clear supernatant fluid was removed (to within 0.5 cm. of the packed sediment, which left less than 0.4 cc. of fluid), and the sediment was resuspended in the original volume of saline, with result in a suspension quite as turbid as before centrifugation. 8 cc. of the clear supernatant fluid was next centrifugalized in a lusteroid tube (internal diameter 1.25 cm., overall length 7.5 cm.) at about 18,000 R.P.M. for 6 hours, in an International centrifuge with high speed conical head attach-

TABLE XIV

Complement Fixation and Pathogenicity Tests with a Centrifugalized Extract of Infectious Papillomas

| | Papilloma extract | | Pathogenicity tests* | | | | | | Complement fixation test† | | | | |
| | | | 5 per cent suspensions Test rabbits | | | 1 per cent suspensions Test rabbits | | | Dilution of extract | | | | |
Centrifugation	Material used	Gross character	A	B	C	A	B	C	1:20	1:40	1:80	1:160	1:320
(a) 910 r.p.m., 5 min.	Supernatant	Turbid	+++++	+++++	+++++	++++±	++++	+++±	+++++	+++++	+++++	+++++	++++±
(b) 4000 r.p.m., 20 min.	Supernatant	Faintly opalescent	+++++	+++++	+++++	++++±	+++	++++±	+++++	+++++	+++++	+++++	++++±
	Sediment resuspended	Turbid, like (a)	++±	+±	++±	+	±	0	0			0	
(c) 18,000 r.p.m., 360 min. (portion of the supernatant of (b))	Supernatant	Water-clear	0	0	0	0	0	0	0	0	0	0	0
	Sediment resuspended	Faintly opalescent	+++++±	+++++±	+++±	++++±	+++	+	++++±	+++++	0	0	0

Immune serum W.R. 55, diluted 1:4.

Serum and antigens showed no anticomplementary effect when tested in double amount.

* Readings made on the 20th day after inoculation.

† 2 units of complement in all tubes.

729

ment, used through the courtesy of Dr. Albert Claude. The final high speed centrifugation deposited a considerable amount of brownish sediment, the supernatant fluid becoming absolutely water-clear. The latter was removed, down to within 0.5 cm. of the sediment, and sufficient saline added to resuspend the sediment in 8 cc. Much of the brownish deposit remained caked and could not be resuspended, but some of it was resuspended readily, causing a barely perceptible opalescent sheen.

The various materials thus procured were tested for pathogenicity and complement-binding capacity as usual. Table XIV shows the results. The turbid fluid obtained after centrifugation at 940 R.P.M. was highly pathogenic, and it bound complement in high titer. The clear supernatant fluid obtained after centrifugation at 4000 R.P.M. for 20 minutes was likewise highly pathogenic, and it bound complement practically as well as the crude, turbid extract. The cloudy suspension resulting from the resuspension of the sediment deposited at 4000 R.P.M. showed no complement-binding capacity in the test, although there was a small amount of the virus in it, as the pathogenicity tests showed. The water-clear supernatant fluid obtained after centrifugation at 18,000 R.P.M. for 6 hours was likewise devoid of complement-binding capacity, and it was practically free from virus, no lesions appearing by the 20th day after it was inoculated into 3 rabbits, though a few discrete lesions made their appearance on the 27th day. The resuspended sediment contained virus in large amount and it bound complement in dilutions equivalent to 1:20 and 1:40 of the original extracts. It is apparent that the virus was sedimented, though not completely, by the high speed centrifugation.

The experiment shows plainly (Table XIV) that the complement-binding antigen was present in the crude and centrifugalized extracts of the papillomas, and in much the same proportions as the virus. It was sedimented by high speed centrifugation, as was the virus, the supernatant fluids being thus rendered practically free from the latter and having no detectable capacity to bind complement. The findings yield no hint that aggregated material played any significant part in the findings, for the supernatant fluid, rendered practically water-clear by centrifugation at 4000 R.P.M. in the angle head centrifuge, contained as much virus as the turbid, crude extract, and it bound complement as well; whereas the crude sediment, resuspended in saline, showed no complement-binding capacity and very little pathogenicity.

Effect of Heat on the Complement-Binding Antigen and the Virus

Shope has demonstrated that the papilloma virus is notably resistant to heat (3). Does the complement-binding antigen share this attribute?

Experiment 15.—5 per cent suspensions of the highly infectious, glycerolated warts of W.R. 8-N and W.R. Tx were centrifugalized twice and filtered through Berkefeld V candles, after having stood overnight in the refrigerator. Portions were put into the bottom of a series of test tubes without touching the sides. The tubes were then corked tightly and submerged almost completely in water baths at 56°, 60°, 63°, 66°, and 69°C. for 30 minutes. The heated filtrates, and the un-

TABLE XV

The Effect of Heat on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests[*]			Complement fixation tests
Source	Temperature (30 min.)	Test rabbits			Immune serum D.R. 7
		A	B	C	
	°C.				
W.R. 8-N	Unheated	++++	++++	+++	
	56				+++
	60	++++	++++	+++	++++
	63	0	0	0	0
	66	±	0	0	0
	69	0	0	0	0
W.R. Tx	Unheated	++++	++++	+++	
	56				++++
	60	++++	++++	+	++++
	63	0	0	0	0
	66	0	0	0	0
	69	0	0	0	0

Serum diluted 1:4.
Papilloma extracts, 1:20.
None of the extracts was anticomplementary when tested concurrently in double amount, nor was the immune serum.
[*] Readings made on 36th day after inoculation, according to the standard scale.

heated portions, were then compared for pathogenicity and complement-binding capacity.

In Shope's experiments, the papilloma virus withstood temperatures of 65–67°C. when heated in crude suspension for 30 minutes. The filtrates of the present experiment, however, were inactivated at 63°C. (Table XV), with a single exception,—a discrete growth arising in test rabbit A, where the W.R. 8-N filtrate heated at 66° had been inoculated.

The findings (Table XV) show that heating at 56° and 60° had no noteworthy effect on either the virus or the complement-binding antigen, whereas 63°, 66°, and 69°C. rendered both inactive.

Effect of Ultraviolet Light

Ultraviolet light is known to inactivate viruses generally, some of them soon, others only after considerable exposure. Comparative tests were now made of its effects on the papilloma virus and the complement-binding antigen. Extracts of the papillomas were exposed to ultraviolet light for various periods, and their infectious and complement-binding capacities later determined.

An unreported experiment done several years ago for other purposes had shown the papilloma virus to be notably resistant to ultraviolet light, a potent 1 per cent Berkefeld V filtrate of W.R. 1240 virus withstanding irradiation for 20 to 30 minutes under conditions inactivating the Shope fibroma virus in 5 minutes.

Experiment 16.—5 per cent extracts of the glycerolated, naturally occurring papillomas of 2 cottontails were prepared and centrifugalized at 3500 R.P.M. for 15 minutes. 3 cc. aliquots of the slightly opalescent, supernatant fluids were then irradiated for various periods in open Petri dishes, in which they formed a layer about 2 mm. deep. The Petri dishes were placed directly beneath a mercury vapor lamp[1] at a distance of 48 cm., and each was shaken and turned round at frequent intervals throughout the irradiation. The temperature of the air about the irradiated fluids did not rise above 28°C. There was some evaporation (up to 1.5 cc.) from the fluids irradiated for the longer periods, and sufficient saline was added immediately after the irradiation to restore their bulk. Complement fixation and pathogenicity tests with the irradiated and control fluids were carried out in the usual ways. The results are recorded in Table XVI. One of the 3 test rabbits died 2 days after inoculation, and the 2 remaining showed a rather pronounced difference in susceptibility to the virus. From their lesions (recorded in the table on the 42nd day) it is obvious that the pathogenicity of the extracts was not notably diminished by irradiation for 10 minutes, but was progressively reduced by longer exposures and completely abolished by irradiation for from 60 to 90 minutes. The single discrete growth, arising on the most susceptible rabbit where the material of W.R. 56 irradiated for 120 minutes had been inoculated, was most probably due to the chance survival of a single infectious entity that had not received its full quota of irradiation. In contrast to the loss of pathogenicity, the complement-binding capacity of the irradiated materials was not reduced a whit, even after irradiation for 120 minutes.

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[1] Alpine sun lamp, Hanovia Chemical Company.

The findings show (Table XVI) that ultraviolet light can render papilloma extracts non-pathogenic without diminishing their capacity

TABLE XVI

The Effect of Ultraviolet Light on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests		Complement fixation tests						
Source	Irradiation with ultraviolet light, 48 cm.	Test rabbits		Immune sera		Hyperimmune sera		Normal sera		Antigen controls (no serum)
		A	B	15	55	8-S	12-S	30	36	
	min.									
W.R. 54	Nil	++++	+++	++++	++++	++++	++++	0	0	0
	10	++++	++±	++++	++++	++++	++++	0	0	0
	20	++++	±	+,+++	++++	++++	++++	0	0	0
	30	++++	±	++++	+,+++	++++	++++	0	0	0
	45	++±	±	++++	++++	++++	++++	0	0	0
	60	+++	+	++++	++,++	++++	++++	0	0	0
	90	+	0	++++	++++	++++	++++	0	0	0
	120	0	0	++++	++++	++++	++++	0	0	0
W.R. 56	Nil	++++	+++	++++	++,++	++++	++++	0	0	0
	10	+++±	+++	++++	++++	++++	++++	0	0	0
	20	++++	+±	++++	++++	++++	++++	0	0	0
	30	++++	+	++++	++++	++,++	++++	0	0	0
	45	+++	±	++++	++++	++++	++++	0	0	0
	60	+++	+	++++	++++	++++	++++	0	0	0
	90	++	0	++,++	++++	++++	++++	0	0	0
	120	±	0	++++	++++	++++	++++	0	0	0
Serum controls (no antigen).....		0		0	0	0	0	0	0	

Complement, 2 units in all tubes.
Sera diluted 1:4.
Papilloma extracts, 1:20.

to bind complement,—a finding that parallels the work of others with different virus materials (4).

Effect of Changes in Hydrogen Ion Concentration

The papilloma virus is known to be inactivated rapidly at certain pH levels, slowly at others (5). In the next experiment the effect of

TABLE XVII

The Effect of Hydrogen Ion Concentration on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests*			Complement fixation tests						
		Test rabbits			Immune sera		Hyperimmune sera		Normal sera		Antigen controls (no serum)
Source	pH	A	B	C	55	56	8	12	30	36	
W.R. 54	1.8	0	0	0	0	0	0	0	0	0	0
	2.8	0	0	0	0	0	0	0	0	0	0
	4.4	++++	++	++++	++++	++++	++++	++++	0	0	0
	6.6	++++	++++	++++	++++	++++	++++	++++	0	0	0
	9.0	+++	++	+++	++++	++++	++++	++++	0	0	0
	9.8	0	0	0	0	±	0	0	0	0	0
	10.5	0	0	0	0	0	0	0	0	0	0
W.R. 56	1.8	0	0	0	0	0	0	0	0	0	0
	2.8	0	0	0	0	0	0	0	0	0	0
	4.4	++++	+++	+++	+++	+++	+++	+++	0	0	0
	6.8	++++	++++	++++	++++	++++	++++	++++	0	0	0
	9.0	+++	+++	+++	+++	+++	+++	+++	0	0	0
	9.8	±	±	±	0	±	±	±	0	0	0
	10.5	0	0	0	0	0	0	0	0	0	0
Serum controls (no antigen)					0	0	0	0	0	0	

Complement, 2 units in all tubes.

Sera diluted 1:4 in tests with antigen, 1:20 in controls.

Papilloma extracts, 1:2 in controls. They were kept at the stated pH levels for 14 hours at 37°C., then brought back to pH levels of 6.6 and 6.8 and tested.

* Lesions recorded on the 28th day.

changes in hydrogen ion concentration on the complement-binding capacity and the pathogenicity of papilloma extracts was determined.

Experiment 17.—The pH of 10 per cent centrifugalized extracts prepared in saline as usual of the glycerolated, naturally occurring papillomas of 2 cotton-tails (54 and 56), was found to be respectively 6.6 and 6.8. 2 cc. aliquots of these extracts were brought to pH levels of 1.8, 2.8, 4.4, 9.0, 9.8, and 10.5,—as determined by indicator dyes,—by the addition of 0.1 to 0.2 cc. of appropriate dilutions of normal HCl or NaOH. These were incubated, along with portions of the untreated extracts, for 14 hours at 37°C., and then all were adjusted to pH 6.6 to 6.8. Sufficient saline was added next to bring the final dilution of the extracts to 1:40. The fluids were then tested for pathogenicity and complement-binding capacity (Table XVII).

At pH levels of 1.8, 2.8, and 10.5 the pathogenicity and complement-binding capacity of the materials were abolished (Table XVII), while both remained unaltered at pH 4.4 and 9.0, as also at 6.6 and 6.8. At pH 9.8, however, the extracts had lost completely or almost completely their infectious properties, while retaining undiminished their capacity to bind complement. It is of interest to compare these findings with those of Svedberg and his associates (6), who showed that certain proteins tend to become unstable at the extremes of their pH stability ranges, and with the findings of Wyckoff and Beard (5), who found that the active material that can be isolated from extracts of infectious papillomas by centrifugation is fragmented at pH levels below about 3.0 and above about 10.2, while it remains intact at hydrogen ion concentrations in-between. In view of their findings and ours it is reasonable to suppose that the infectivity and the antigenicity of the active material in extracts of the papillomas depends upon its integrity, and that this is destroyed by heating to 63–67°C., or by treating with acid or alkali in pH ranges below about 3.0 or above about 10.0. In this relation the fact assumes importance that the pathogenicity of a virus material can be abolished by irradiation with ultraviolet light and by treatment with weak alkali without destroying its antigenicity.

SUMMARY AND COMMENT

The antigen that binds complement in the presence of sera neutralizing the Shope papilloma virus can be readily extracted from

papillomas yielding infectious virus, but not from the normal skin of rabbits bearing the growths. The virus and the complement-binding antigen appear to have the same particle size, as determined by filtration, and they are thrown down together in the centrifuge. They are destroyed by the same amounts of heating and, in general, by the same changes in pH. It is possible, nevertheless, by irradiation with ultraviolet light, or by treatment with weak alkali, to render papilloma extracts non-pathogenic without diminishing their capacity to bind complement when mixed with immune serum.

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IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS

III. Antigenicity and Pathogenicity of Extracts of the Growths of Wild and Domestic Species: General Discussion

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The Shope virus engenders papillomas when inoculated into domestic rabbits as well as cottontails, yet only rarely can it be recovered from these (1), and occasionally the virus-induced growths of cottontails fail to yield it. Yet serological studies have indicated that the virus exists in the growths from which it cannot be got, causing their continued proliferation, and eliciting antibodies roughly in proportion to the amount of papillomatosis present on the individual (2). As further evidence of the presence of the virus, Shope has recently found that suspensions of the virus-induced papillomas of wild and domestic rabbits will elicit virus-neutralizing antibodies when inoculated intraperitoneally into rabbits of homologous species, even though they cause no lesions when inoculated into the skin of susceptible animals (3).

The experiments now to be reported were done to learn more about the state of the virus, and of the complement-binding antigen, in the papillomas that yield no infectious virus on extraction. After they have been reported, the findings of all three papers will be discussed.

Complement Fixation Tests with Extracts of Non-Infectious Papillomas of Cottontails

A number of instances have been encountered in our laboratory in which large, flourishing papillomas, brought about by the experimental inoculation of Shope virus into highly susceptible cottontails, failed to yield virus on extraction. The experiment that follows was done to determine whether extracts of such growths will bind

complement when tested with suitable antisera. 6 extracts of growths that were known from previous tests to yield little or no active virus were compared as to complement-binding capacity and pathogenicity with 6 others containing virus in high titer.

Experiment 18.—1:20 saline extracts of the growths, which had been in 50 per cent glycerol for periods up to 30 weeks,[1] were prepared as usual, all in the same way, and centrifugalized twice at 3500 R.P.M. in the angle head centrifuge. The supernatant fluids were then tested for complement-binding capacity according to the method adopted as standard (Paper I), and for pathogenicity by inoculation into scarified areas on the skin of 3 normal domestic rabbits, according to a routine method (2).

Table XVIII, giving the results of the experiment, shows that the extracts that contained active virus in large amount bound complement notably well, while those containing little or no virus fixed it poorly, or not at all.

The Yield of Virus from the Natural and Experimental Papillomas of Cottontails

How does it happen that the virus-induced growths of some cottontails yield the virus and the complement-binding antigen in high titer, while others furnish neither in significant amount? The different amounts of virus obtained from growths of various duration, size, and character provide data bearing upon this point.

The naturally occurring papillomas are generally small, discrete, conical or discoid, superficial growths, 1 to 2.5 cm. across at the base, with horny peaks 1 to 3 cm. high. They are frequent, being present on one of every dozen or so cottontails trapped in certain Western states; and, though often multiple, are not usually numerous on the individual. Only occasionally can more than a few grams of papilloma tissue be obtained from a single animal, our maximum being 24.5 gm., although Shope, with wider experience, has sometimes obtained more.[2]

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[1] The preservation of papillomas in glycerol for many months does not harm the virus perceptibly, as Shope first noted; and materials preserved in this way in our laboratory for more than 2 years have retained their pathogenicity without any notable loss in titer. Indeed an apparent increase in the yield of virus has been observed in some cases when the growths had been kept long in glycerol at refrigerator temperature.

[2] Personal communication from Dr. Shope.

TABLE XVIII

Complement Fixation Tests with Extracts of Papillomas Yielding Much Virus and of Others Yielding Little or None

Source of papilloma extract	Pathogenicity tests* (Test rabbits)			Complement fixation tests								
				Immune sera				Hyperimmune sera		Normal sera		Antigen controls (no serum)
W.R. No.	A	B	C	53	56	15	19	8-S	12-S	31	35	
35	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0	0
36	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0	0
45	+++	+++±	+++±	+++	+++	+++	+++	+++	+++	0	0	0
1-30	+++	+++±	+++	+++	+++	+++	+++	+++	+++±	0	0	0
27	+++	+++±	+++±	+++±	+++±	+++±	+++±	+++±	+++±	0	0	0
20	+++	+++	+++	±	0	+++	+++	+++	+++	0	0	0
1-15	0	0	0	0	0	0	+++	+++	±	0	0	0
1-26	0	0	0	0	0	0	±±	+++	0	0	0	0
14	0	0	0	0	0	0	0	±	0	0	0	0
19	0	±	±	0	0	0	0	±	0	0	0	0
15	±	±	0	0	0	0	0	+	0	0	0	0
37	0	0		0	0	0	0	0	0	0	0	0
Serum controls (no antigen)				0	0	0	0	0	0	0	0	0

Complement, 2 units in all tubes.

Antigen extracts diluted 1:20, sera 1:4.

* Readings made on 42nd day after inoculation of 5 per cent suspensions into test rabbits, according to standard scale,—see Table XIII.

TABLE XIX

The Yield of Virus from the Naturally Occurring and Experimentally Induced Papillomas of Cottontail Rabbits

Cottontail rabbit No.	Type of growths	Character and size of growths	Day growths procured after virus inoculation	Weight of papilloma tissue carried by the animal	Pathogenicity tests with extracts of the growths								
					15 days			25 days			42 days		
					A	B	C	A	B	C	A	B	C
W.R. 24	Experimental	Large, fleshy, confluent, 6 x 4 cm. on both flanks, 3 cm. high, with underlying extensions	83rd	*gm.* 87.5	0	0	0	0	0	0	±	±	0
25	"	Large, fleshy, confluent, 6 x 4 cm. on both flanks, 3½ cm. high, with "satellite pearls"	111th	107.5	0	0	0	0	0	0	0	±	0
60	"	Large, vigorous, confluent and semiconfluent growths on abdomen	74th	43.5	0	0	0	±	0	0	0	±	0
57	"	Large, vigorous, confluent growth on abdomen, 6 x 4 cm., 1 cm. high	60th	23.0	0	0	0	±	0	0	0	±	0
20-S	"	Large, confluent and semiconfluent growths 6 x 5 cm. on both flanks, 1.5 cm. high	80th	71.2	+	0	++	+++±	+++	+++	++++±	+++	+++
7-S	"	Large, confluent, fleshy, 6 x 5 cm. on both flanks, 0.8 cm. high	28th	43.2	+	+	++	+++±	++	+++±	+++	+++	+

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	Description	Week		1	2	3	4	5	6	7	8	9
18-S	"	Large, confluent, fleshy, 6 x 4 cm. on both flanks, 1.4 cm. high	28th	47.5	+	++	0	+++	+++	++±	+++	++±	+++
4-S	"	Large, confluent, fleshy, 6 x 5 cm. on both flanks, 1.0 cm. high	20th	34.8	++	++	++	+++	+++	+++	+++	+++±	+++±
10	Natural	8 discrete, fleshy, discoid papillomas 2 to 3 cm. across and 1 to 2 cm. high	Unknown	17.4	+++	+++	±±	+++++	+++	+++	++++	++++	++++
9	"	9 discrete, fleshy, discoid growths 2 to 3 cm. across and 1 cm. high	"	14.4	+++++	+++++	+++±	+++++	++++±	++++	+++++	+++++	++++±
8	"	7 discrete, fleshy, discoid growths 1 to 3 cm. across and 1 to 2 cm. high	"	10.5	+++++	+++++	++	+++++	+++	+++	+++++	+++++	+++±

Readings made according to the standard scale,—see Table XIII.

A, B, C = test rabbits.

Dilution of extracts, 1:20, except W.R. 60 and 57, 1:10.

The size of experimentally induced growths depends on the area inoculated. As a rule, we have rubbed large amounts of potent virus into broad areas of scarified skin, and the resulting massive growths have frequently weighed from 75 to 100 gm. or more.

Table XIX gives a synopsis of the findings with extracts of the growths of 3 cottontails with naturally occurring papillomas and 8 others bearing experimentally induced growths. All of the growths had been procured some months previously while the rabbits were alive. They were at once cut up and put into 50 per cent glycerol-Locke's solution in the refrigerator. Tests for pathogenicity were made on three separate occasions, each with 3 test rabbits. 5 per cent extracts of the experimentally induced papillomas of W.R. 24, 25, 20-S, 7-S, and 18-S were tested on one series of rabbits, 10 per cent extracts of the growths of W.R. 60 and 57 on a second series, while 5 per cent extracts of the naturally occurring growths of W.R. 8, 9, and 10 were tested in a third experiment. The susceptibility of the test animals varied little in the three experiments, and hence the readings have been brought together in the table. All of the materials, except those of W.R. 7-S and 18-S, were later tested in other experiments using other test animals, and the results agreed closely with those set down in Table XIX.

Extracts of the glycerolated materials from 4 of the experimentally inoculated rabbits (W.R. 24, 25, 60, and 57), which had carried large, confluent growths over periods up to 111 days, contained little if any active virus (Table XIX); while similar extracts of the growths of individuals that had carried experimentally induced papillomas for shorter periods (W.R. 20, 7-S, 18-S, and 4-S) contained virus of moderate pathogenicity. Extracts of the small, discrete, naturally occurring papillomas of W.R. 8, 9, and 10, on the other hand, contained virus of high titer, the materials all giving rise early to confluent papillomas. Individual host differences have an evident rôle in the findings, however: the experimental growths of W.R. 20-S yielded much more virus than those of W.R. 57 and 60, though their total mass was greater, and they had been present for a longer period.

The findings set down in Table XIX provide a fair sample of our general experience, which is to the effect that the small, discrete, naturally occurring papillomas of cottontail rabbits usually furnish virus of high titer; while the larger, confluent growths, produced with it experimentally in highly susceptible cottontails, yield it in moderate or small amounts or not at all.[3]

In tests already recorded (Tables III and X of Papers I and II), the sera of cottontail rabbits with large, experimentally induced growths have had high antibody titers. It is precisely such growths

[3] Shope has also noted these facts, incidentally to the routine titration of papilloma materials (personal communication).

that yield the least virus. Have the two findings any relation to one another? To learn about this, the growths of 2 cottontails with different antibody titers were compared as to their yield of virus and complement-binding antigen.

Experiment 19.—2 normal cottontail rabbits were inoculated by rubbing a 10 per cent extract of the growths of W.R. 7-N into scarified abdominal areas about 8 x 10 cm. across. The growths of one of the inoculated animals (W.R. 27) appeared sooner than those of the other (W.R. 26) and they became much larger, fleshier, and more numerous. On the 87th day after inoculation both rabbits were bled from the heart to secure serum, and the growths of each were pulled away and stored in 50 per cent glycerol in the refrigerator. Those of W.R. 27 were large, confluent and semiconfluent, fleshy, compact and onion-like, and they covered most of the broad, inoculated area. Their greatest height was 1.5 cm., and beneath them were a number of rounded "pearls" like those often formed by the deep extension of vigorous papillomas in cottontails. The growths of W.R. 26, although caused by the same strain of virus, were by contrast much smaller and fewer, discrete but fleshy "onions," scattered thinly over the inoculated site, and raised only 1 cm. or less above the skin level. The mass of papilloma tissue obtained from W.R. 27 was 49.8 gm., and from W.R. 26, 9.9 gm.

Representative portions of the glycerolated papilloma tissues of the 2 animals were extracted as usual (dilution 1:20), and the extracts tested for pathogenicity and complement-binding capacity. The complement fixation and virus neutralization titers of their sera were also determined.

The findings are shown in Table XX. The extract of the papillomas of W.R. 26 contained virus in high titer, confluent and semiconfluent growths appearing before the 15th day where it had been inoculated into all 3 test rabbits; but no growths ever appeared where the extract of the papillomas of W.R. 27 was inoculated. The extract of the growths of W.R. 26, containing virus in high titer, bound complement with all of the immune sera; while that of W.R. 27, devoid of pathogenicity, failed to bind complement under the same conditions. The sera of the 2 cottontails showed wide differences, that of W.R. 26 neutralizing a potent 5 per cent virus extract in low titer and binding complement poorly on test with 2 antigens, while the serum of W.R. 27 neutralized the virus in very high titer and fixed complement correspondingly.

This experiment proved highly informative. The large, confluent, fleshy papillomas of one animal yielded no active virus whatever. and the serum of this individual had great power to neutralize virus and to bind complement. Precisely the opposite state of affairs existed in the other rabbit, the growths being small, scattered, and slow growing, though engendered by the same inoculum, and the

The Yield of Virus and of the Complement-Binding Antigen from the Papillomas of Two Cottontail Rabbits with Different Serum Antibody Titers

TABLE XX

Cottontail rabbit No.	Day serum and growths procured	Number and character of papillomas	Weight of papilloma tissue carried by the animal	Tests with extracts of the papillomas					Tests with the sera							
				Pathogenicity tests*			Complement fixation tests†		Neutralization tests‡			Complement fixation tests§				
							Immune sera		5 per cent virus W.R. 56+			Dilutions of serum				
				15th day	24th day	42nd day	53	56	Whole serum	Serum 1:4	Serum 1:16	1:4	1:8	1:16	1:32	1:64
W.R. 26	87	Few, small, scattered, discrete, fleshy, less than 1 cm. high	9.9 gm.	+++	++++±	++++	++++	+++	+++	±	0	±	0	0	0	0
27	87	Many large, confluent and semiconfluent, fleshy, compact, 1.5 cm. high	49.8	0	0	0	0	0	++++	++++	++++±	++++	++++	++++	++++±	0

* 5 per cent extracts. +++ = semiconfluent papillomatosis. ++++ = confluent papillomatosis; ++++ = complete fixation of 2 units of complement.
† 5 per cent extracts. Immune sera diluted 1:4. ++++ = complete neutralization. Immune sera
‡ Serum-virus mixtures incubated 2 hours at 37°C. and inoculated in 3 test rabbits. The readings given are those of one rabbit on the 20th day.
§ Antigen = 1:10 Berkefeld V filtrate of papillomas of W.R. 35. ++++ = complete fixation. The antigen and sera were not anticomplementary when tested in double amounts.

serum having little neutralizing power and none to bind complement.

When considered together, the findings in this experiment and in those of Tables XVIII and XIX suggest strongly that the failure of large, confluent papillomas to yield virus and complement-binding antigen may be related to the fact that the sera of animals carrying the large growths develop marked ability to neutralize the virus; but further tests must be made to settle the point definitely. However this may be, it is certain that the yield of the complement-binding antigen parallels the yield of virus from the papillomas of cottontails,— some growths providing much of the one and furnishing much of the other also, while others yield little or none of either.

Complement Fixation Tests with Extracts of Virus-Induced Papillomas of Domestic Rabbits, Snowshoe Hares, and Jack Rabbits

As already stated, the papilloma virus gives rise to growths of exceptional vigor when inoculated into the skin of domestic rabbits, yet only exceptionally can it be recovered from them, and then in attenuated state.[4] Many tests have been made in the course of the present work to determine whether extracts of virus-induced papillomas of domestic rabbits have the capacity to bind complement. The results make it clear that they are far inferior to cottontail materials in this respect, having little or no capacity of the sort. An illustrative experiment will be given.

Experiment 20.—Extracts were made in saline as usual of the glycerolated papillomas of 4 cottontail rabbits, known to yield the virus in high titer, and from the glycerolated growths of 4 domestic rabbits known from previous tests to yield virus in very small amount or not at all. After standing overnight in the refrigerator the extracts were centrifugalized twice as usual at 3500 R.P.M., and tested in dilutions of 1:5, 1:15, and 1:45 for capacity to bind complement in the presence of various dilutions of the hyperimmune serum of W.R. 8-S.

Table XXI shows the results of the experiment. Most of the antigens proved anticomplementary in a dilution of 1:5 when tested in

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[4] Shope has procured several strains of virus that can be passed serially in domestic rabbits; but all of them are slightly or moderately pathogenic at best, giving only scattered or semiconfluent growths that appear long after inoculation (1).

double amount, and hence the findings with these dilutions have been omitted from the table. 3 of the 4 extracts from the cottontail materials bound complement notably well, while the fourth (W.R. 30-N)

TABLE XXI

Complement Fixation Tests with Extracts of Papillomas from Domestic and Cottontail Rabbits

Source of antigens	Rabbit No.	Dilutions of antigen	Complement fixation tests Dilutions of serum			Antigen controls (no serum)
			1:5	1:20	1:80	
Cottontail rabbits (Papillomas yielding active virus)	10	1:15	++++	++++	++++	
		1:45	++++	++++	++++	0
	8-N	1:15	++++	++++	+++	
		1:45	++++	++++	+++	0
	55	1:15	++++	++++	++	
		1:45	++++	++++	0	0
	30-N	1:15	0	0	0	
		1:45	0	0	0	0
Domestic rabbits (Papillomas yielding little or no active virus)	K-22	1:15	++++	+±	0	
		1:45	0	0	0	0
	55-N	1:15	0	0	0	
		1:45	0	0	0	0
	12-85	1:15	0	0	0	
		1:45	0	0	0	0
	30-N	1:15	0	0	0	
		1:45	0	0	0	0
Serum controls (no antigen)..............			0	0	0	0
Serum W.R. 8-S (hyperimmune).			0	0	0	

Complement, 2 units in all tubes.

failed to do so in the dilutions shown, although it gave complete, specific fixation at a dilution of 1:5, and on the other occasions at 1:10. Only one of the materials from the domestic rabbits bound complement, and this was derived from growths that had yielded a small

amount of infectious virus on a number of previous trials. The other 3 extracts failed to show any specific complement-binding capacity at all. One of these (D.R. 12-85) had yielded virus in small amount on previous extractions, while the others (D.R. 55-N and 30-N) had proven non-infectious in all tests.

The papilloma virus will produce growths when inoculated into snowshoe hares and jack rabbits (4), and from them it can often be recovered, but in low titer. In the next test extracts of papillomas experimentally induced in 2 snowshoe hares and a jack rabbit were tested for capacity to bind complement in the presence of a known immune serum. Extracts of the papillomas of a domestic rabbit and of a cottontail were used for comparison.

Experiment 21.—The glycerolated papilloma material procured from the 2 snowshoe hares and from the jack rabbit had yielded infectious virus in small amount on several previous tests, while extracts of the warts of the domestic rabbit had proven non-infectious. The papillomas of the cottontail (W.R. 1240) had furnished large quantities of virus in many previous tests. For present purposes extracts of all 4 glycerolated materials were prepared as usual and tested with various dilutions of a potent immune serum.

The only extract that bound complement notably well in the test (Table XXII) was that of the cottontail growth, a material containing active virus in large amount. The extracts of the jack rabbit papillomas, although somewhat anticomplementary, exhibited some specific fixation, as did the extracts of the growths from the snowshoe hares and from the domestic rabbit, though none gave complete fixation.

The findings should be considered with those of Tables XIX and XXI. Together they show that extracts of the virus-induced papillomas of domestic rabbits, snowshoe hares, and jack rabbits, which contain infectious virus in small amount or not at all, have little or no complement-binding capacity.

Does the Complement-Binding Antigen Exist in Masked Form in the Non-Infectious Growths of Domestic Rabbits?

Shope has found that extracts of the "non-infectious" growths of domestic and cottontail rabbits elicit virus-neutralizing antibodies when injected intraperitoneally into rabbits of homologous species

(3). He has generously provided for complement fixation tests a number of virus-neutralizing sera obtained from rabbits immunized by intraperitoneal injections of papilloma extracts, some of which contained active virus while others did not. The sera bind complement in the presence of suitable antigens, as is shown by the tests that follow.

TABLE XXII

Complement Fixation Tests with Extracts of Papillomas from Various Species of Rabbits

Source of antigen	Rabbit No.	Dilutions of antigen	Complement fixation tests			
			Immune serum D.R. 9			Normal serum D.R. 1
			1:2	1:4	1:8	1:2
Cottontail rabbit.........	1240	1:15	++++	++++	++++	
		1:45	++++	++++	++±	0
Jack rabbit..............	6	1:15	+++±	+++±	++±	
		1:45	++	±	±	+±
Snowshoe hare..........	1	1:15	++	±	±	
		1:45	±	0	0	0
Snowshoe hare..........	2	1:15	+++±	+++	+++±	
		1:45	+	±	±	±
Domestic rabbit.........	55	1:15	+++±	++±	+	
		1:45	+	0	0	0
Serum controls (no antigen).............		1:15	++±	++±	+	
		1:45	+	0	0	0
2 units of complement in all tubes.			0	0	0	0

Experiment 22.—3 of the sera (14-04, 14-05, and 14-06) were from domestic rabbits immunized by intraperitoneal injections of suspensions of the non-infectious virus-induced papillomas of a domestic rabbit, 3 (14-07, 14-08, and 14-09) from rabbits similarly inoculated with suspensions of the slightly infectious papillomas of a 2nd domestic rabbit, and 3 (14-10, 14-11, and 14-12) from rabbits immunized with suspensions of the highly infectious papillomas of a cottontail rabbit. All were tested in a dilution of 1:4 with a potent antigen according to the routine technique, the sera of 3 of the rabbits procured before the immunizing injections being used as controls (Table XXIII).

In a quantitative test with the sera of 6 of these rabbits (Table XXIV) it was found that those immunized with suspensions of cottontail papillomas, which contained much virus, bound complement in much higher titer than those immunized with suspensions of the domestic rabbit growths, which contained no active virus. The differences

TABLE XXIII

Complement-Binding Capacity of the Sera of Domestic Rabbits Immunized by Shope by Means of Intraperitoneal Injections of Suspensions of Papilloma Tissue

	Source of serum		Complement fixation tests	
	Immunization (2 intraperitoneal injections of 5 per cent suspensions of glycerolated papillomas)			
Rabbit No.	Source of immunizing material	Amount of active virus in immunizing material	Antigen W.R. 8-N 1:40	Serum controls (no antigen)
14-04	Before injection	—	0	0
14-05	"	—	0	0
14-06	"	—	0	0
14-04	Domestic 12-86	None	±	0
14-05	"	"	++++	0
14-06	"	"	++++	0
14-07	Domestic 12-70	Little	++++	0
14-08	"	"	0	0
14-09	"	"	+++	0
14-10	Cottontail 12-34	Much	++++	0
14-11	"	"	++++	0
14-12	"	"	++++	0
Antigen control (no serum)...........................			0	

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigen, 1:40 extracts of glycerolated papillomas (W.R. 8-N).

correspond closely with those obtained by Shope in neutralization tests with the same sera.

The findings set down in Tables XXIII and XXIV show that an antigen capable of eliciting complement-binding antibodies is present in crude extracts of the "non-infectious" papillomas of domestic

rabbits, though in much smaller amount than in extracts of growths containing the virus. How has it happened, then, that extracts of the "non-infectious" growths have proved ineffective as complement-binding antigens in the tests *in vitro*? Can it be that the antigen, which Shope readily demonstrated in crude suspensions of the "non-infectious" growths, is eliminated by the centrifugation or filtration procedures used as routine in the preparation of antigens for the complement fixation tests? It could be so, for a preliminary effort to

TABLE XXIV

Complement Fixation Tests with the Sera of Domestic Rabbits Immunized by Intraperitoneal Injections of Crude Extracts of Domestic and Cottontail Papillomas

Source of serum		Complement fixation tests			
Domestic rabbits	!D.R. No.	Dilutions of sera			Serum controls (no antigen)
		1:4	1:6	1:8	
Immunized with suspensions of D.R. papillomas containing no active virus	14-07 14-08 14-09	+ 0 +	0 0 0	0 0 0	0 0 0
Immunized with suspensions of W.R. papillomas containing much virus	14-10 14-11 14-12	++++ ++++ ++++	++++ ++++ ++++	++++ ++++ ++++	0 0 0
Antigen control (no serum).............		0			

Complement, 2 units in all tubes.
Antigen, 1:20 extract of cottontail papillomas (W.R. 8-N).

The sera were generously provided by Dr. Shope. His experiments showed that the sera of the rabbits immunized with suspensions of wild rabbit papillomas neutralized the virus considerably better than those immunized with suspensions of domestic rabbit growths (3).

stimulate the formation of complement-binding antibodies by repeated intravenous injections of large quantities of Berkefeld V filtrates of the "non-infectious" papillomas of domestic rabbits yielded negative results. Consequently resort was had to the intraperitoneal injection of crude suspensions as well as Berkefeld V filtrates of the same domestic rabbit papillomas to learn whether the immunizing antigen in the "non-infectious" extracts is retained by Berkefeld filters.

Experiment 23.—10 gm. of the papillomas of 3 domestic rabbits, which had been preserved in glycerol for many months in the refrigerator and had failed to yield infectious virus in a number of previous tests, was ground as usual and made up to 100 cc. with saline. The crude suspension was allowed to settle for 4 hours in the refrigerator, after which the densely turbid supernatant fluid was removed, stirred, and a part set aside for injection as such. The remainder was centrifugalized at 3500 R.P.M. in the angle head for 5 minutes, and the supernatant fluid was spun again at 3500 R.P.M., for 15 minutes. The second supernatant was barely opalescent. It was filtered through a new Berkefeld V 5 candle, coming through rapidly and water-clear. 6 cc. of the crude suspension was injected into the peritoneal cavity of each of 3 normal Dutch rabbits, and 6 cc. of the filtrate into 3 blood-related animals.

Eleven days later the procedure was repeated, using a fresh crude suspension and a V filtrate of the same materials, and injecting the rabbits as before. None developed growths at the sites where the skin had been punctured, or elsewhere. On the 9th day after the second injection the rabbits were bled 20 cc. from the heart, and the sera were tested for capacity to bind complement and to neutralize the virus. Sera from 3 normal Dutch rabbits, and from 3 others bearing large virus-induced papillomas, were tested concurrently for comparison.

As Table XXV shows, the rabbits immunized with the crude extracts of the non-infectious papillomas yielded sera that neutralized completely or partially an active 1 per cent virus filtrate when incubated for 2 hours with it in equal amounts prior to inoculation into the test rabbits. The sera compared favorably in this respect with the specimens procured from animals bearing large papillomas and tested concurrently. The sera of the rabbits injected with filtrates, on the other hand, had no neutralizing capacity whatever. The sera of the animals injected with crude extract failed to bind complement despite their neutralizing power, whereas the sera of the rabbits bearing the growths did so. It will be recalled in this connection that the neutralizing power of the serum of immunized rabbits regularly transcends the complement-binding power, whence one may infer that in the present instance the animals receiving crude extract had developed less immunity than had those bearing growths.

In this experiment (Table XXV) an antigen, present in the crude extracts of "non-infectious" papillomas and capable of eliciting virus-neutralizing antibodies, was removed by centrifugation and filtration, procedures that do not notably affect the content of infectious virus in potent extracts. Even the crude suspensions, though, evoked a relatively low grade of immunity, the sera of rabbits immunized with them neutralizing the virus in low titer, and not binding complement at all.

The next step manifestly was to find out whether the "masked"

antigen, present in crude suspensions of non-infectious growths might not act as a complement-binding antigen *in vitro* if the conditions were rendered suitable. The previous complement fixation tests with

TABLE XXV

Neutralization and Complement Fixation Tests with the Sera of Domestic Rabbits Injected Intraperitoneally with Crude Extracts and Berkefeld V Filtrates of Non-Infectious Domestic Rabbit Papillomas

Source of serum	D.R. No.	Neutralization tests* Serum 0.5 + 1 per cent virus 0.5 Test rabbits			Complement fixation tests Antigens		Serum controls (no antigen)
		A	B	C	W.R. 4-S	W.R. Tx	
Domestic rabbits injected with crude extracts of the non-infectious papillomas	2-58	+±	++±	++	0	0	0
	2-59	++++	++++±	++++	0	0	0
	2-60	++++	++++	++++	0	0	0
Domestic rabbits injected with Berkefeld V filtrates of the non-infectious papillomas	2-63	0	0	0	0	0	0
	2-64	0	0	0	0	0	0
	2-65	0	0	0	0	0	0
Domestic rabbits bearing virus-induced papillomas	2-54	++++	++++	++++	++++	++++	0
	2-74	++++	++++	++++	++++	++++	0
	2-10	++++	++++	++++	++++	++++	0
Normal domestic rabbits	3-04	0	0	0	++++	++++	0
	3-05	0	0	0	0	0	0
	3-06	0	0	0	0	0	0
Antigen controls (no serum).............................					0	0	0

++++ = complete neutralization or complete fixation.
Complement, 2 units in all tests.
Serum 1:2 in all complement fixation tests.
Antigens, 1:20 extracts of glycerolated cottontail papillomas.
* Readings on the 25th day, according to the scale of Table X.

extracts of "non-infectious" papillomas had been done with specimens centrifugalized as usual, and these had bound complement poorly if at all (Tables XXI and XXII). In the next experiment crude suspensions were used, as well as centrifugalized.

Experiment 24.—5 per cent suspensions in saline were made of the highly infectious glycerolated papillomas of 2 cottontails (W.R. 53 and 54), and also of the glycerolated warts of 2 cottontail and 2 domestic rabbits that had failed to yield virus on a number of previous tests. They were left overnight in the refrigerator, then mixed by gentle shaking and allowed to settle for 30 minutes,

TABLE XXVI

Complement Fixation Tests with Crude and Centrifugalized Extracts of Infectious and Non-Infectious Papillomas

Source of antigens			Source of sera					
			Immune		Hyper-immune 8-S	Normal		Antigen controls (no serum)
Papillomas	Rabbit No.	Preparation	D.R. 7	W.R. 10		W.R. 31	D.R. 49	
Yielding much virus	W.R. 53	Crude	+*	++++	++++	0	0	0
		Centrif.	++++	++++	++++	0	0	0
	W.R. 54	Crude	+*	++++	++++	0	0	0
		Centrif.	++++	++++	++++	0	0	0
Yielding little or no virus	W.R. 19	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	W.R. 37	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	D.R. 2-08	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	D.R. 2-11	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
Serum controls (no antigen)........			0	0	0	0	0	0

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigens, dilution 1:20.
* Prozone.

after which 3 cc. of the turbid supernatant fluids were removed for use. The remaining suspensions were now centrifugalized at 3500 R.P.M. for 5 minutes, and the supernatant fluids spun again for 15 minutes. The final fluids thus procured were practically water-clear. They were tested for capacity to bind complement along with the crude extracts, using several immune and normal sera (Table XXVI).

It will be seen that the crude suspensions of "non-infectious" domestic and wild rabbit papillomas failed to bind complement even under the conditions of the test, which were optimal (Table XXVI). If "masked" antigen existed in the crude suspensions, it was evidently present in amount too small or in a form unsuited to the reaction, possibilities that will be considered in the discussion.

GENERAL DISCUSSION

The findings described in the three papers will now be considered under the divisions into which they fall. A recapitulation of the results is essential in this connection.

To begin with a method was worked out that demonstrated the occurrence of complement fixation when the sera of rabbits carrying virus-induced papillomas were mixed with antigens consisting of saline extracts of the growths. The test followed classical lines and yielded consistent results. It proved highly specific. Most, but not all, of the sera of rabbits carrying the virus-induced papillomas fixed complement in the tests, their ability to do so varying roughly in proportion to the total mass of the growth borne by the individual. The sera of normal rabbits, on the other hand, and of rabbits with papillomas elicited by tarring, and of those with various other diseases, including vaccinia and syphilis, gave no complement fixation when mixed with effective antigens derived from the Shope papilloma.

Relation of the Virus-Neutralizing and Complement-Binding Antibodies

In previous work by Shope and ourselves (1, 2) it had been noted that the sera from animals carrying papillomas varied greatly in their virus-neutralizing capacity, some sera having almost none though obtained from rabbits that had borne growths for many weeks. It was of interest, therefore, to find whether the virus-neutralizing capacity of a given serum was proportional to its complement-binding capacity. This proved to be the case. Those sera that neutralized large amounts of the virus fixed complement readily, and often in high titer, their capacity to do so varying directly with their virus-neutralizing capacity; a few sera were encountered, however, that neutralized small amounts of the virus, yet failed to bind complement under the most favorable circumstances.

"Hyperimmunizing" injections were not necessary in order to secure good complement-binding antisera. These were readily obtained from animals that had borne natural or experimentally induced papillomas for some time; and it is reasonable to suppose that growths of long standing elicited "hyperimmune" responses under natural conditions, by liberating antigen more or less constantly over prolonged periods. Complement-binding antibodies could be stimulated in normal rabbits, however, by repeated intraperitoneal injections of extracts of the papillomas, according to the method employed by Shope to elicit virus-neutralizing antibodies (3).

From the findings already discussed it is evident that the complement-binding antibodies are evoked under the same, highly specific conditions as the virus-neutralizing antibodies, and in proportional amounts; and the evidence indicates that they are elicited by the same antigen. The view has been adopted, therefore, that complement fixation and virus neutralization with these sera are probably different manifestations of the action of a single antibody. The neutralization test appears to have a "lower threshold" than the complement fixation test, and smaller amounts of antibody may be detected by means of it; but it is not more sensitive than the complement fixation test in the effective range of the latter, as many experiments have shown.

Relation of the Complement-Binding Antigen to the Virus

A detailed study was next made of the relation of the complement-binding antigen to the virus. It was learned first that the complement-binding antigen is not present in extracts of the normal skin of rabbits, and not in effective amounts in extracts of the papillomas that contain little or no virus. The antigen is always found, on the other hand, in extracts of the growths that contain much virus,—and it varies in amount proportionally to the latter. On further comparison, the virus and the complement-binding antigen appear to have the same particle size; both pass readily through Berkefeld V candles, but are retained in Berkefeld W and Seitz filters. They are also thrown down together in the centrifuge. Furthermore, they are similarly affected by certain physical agents, being inactivated concurrently by heat and, generally speaking, by changes in pH. By irradiation with ultraviolet light, however, it was possible to render

extracts of the growths non-pathogenic without reducing their capacity to bind complement; and mild treatment with alkali attained the same effect,—results that find a parallel in similar tests with other virus materials (5).

The findings provide no hint of the presence of a "soluble antigen" (6), or of any material distinguishable from the virus itself, to account for the immunological activity of extracts of the papillomas. They make it evident that the virus and the complement-binding antigen are closely associated, if not identical; and in so doing they support the view that a single antigen (the virus) elicits both neutralizing and complement-fixing antibodies.

The Phenomenon of "Masking"

In the virus-induced papillomas of most domestic rabbits a singular state exists as concerns the virus. Although produced experimentally with it, and having the same histological character as the infectious growths of cottontails, the papillomas of domestic rabbits usually fail to yield the virus on extraction. The virus manifestly exists in them,—causing the continued proliferation of the papilloma cells, and stimulating the formation of virus-neutralizing and complement-binding antibodies in rising titer as the growths enlarge,—but its pathogenicity and its antigenicity are "masked" (3), even crude extracts failing, as a rule, to induce growths when inoculated into susceptible animals, or to bind complement when mixed with immune serum. The "masking" is not always completely effective though: the growths of some domestic rabbits yield a little virus and a little of the complement-binding antigen on extraction; and crude extracts of the non-infectious growths will usually evoke a slight antibody response when injected intraperitoneally into normal rabbits, though Berkefeld filtrates of the same extracts failed to do so in our experience. It is possible that the "masking" depends in some measure upon an aggregation of the active material, or upon its adherence to "insoluble" cellular constituents; but more extensive tests are necessary before this question can be decided.

"Masking" of the virus is not wholly confined to the papillomas of domestic rabbits. The large, confluent growths produced experimentally in wild rabbits often fail to yield the virus on extraction,

though the discrete, naturally occurring papillomas of cottontails usually furnish it in large amounts. Whether the "masking" of the virus in the large, confluent growths of some cottontails is related to the high antibody titer of the individuals bearing them, or to some other cause, is a question that will be taken up in a subsequent study.

As bearing on the state of the virus in the papillomas of cottontail and domestic rabbits it may be recalled that the sera of cottontails generally have much higher antibody titers than the sera of domestic rabbits with comparable growths. This finding may be adequately explained by assuming that the antigen provided by the growths of cottontail rabbits is more potent or more plentiful than that supplied by the growths of domestic animals. Two facts justify this assumption: the virus and the complement-binding antigen can be readily extracted in large amount from the growths of most cottontails, as not from those of domestic rabbits; and extracts of cottontail papillomas evoke a much greater antibody response than do those of the growths of domestic rabbits when injected intraperitoneally into comparable animals.

General Implications

From the findings as a whole it appears likely that the papilloma virus acts as the antigen that evokes virus-neutralizing and complement-binding antibodies *in vivo*; and that it is closely associated, if not identical, with the antigen that reacts with immune serum to bind complement *in vitro*. It manifestly causes the continued proliferation of the papilloma cells, but without bringing about their death, which takes place through maturation. The virus is evidently liberated in the host over long periods, eliciting "hyperimmune" responses under natural conditions; but there is no evidence that it elaborates "soluble antigens," or that it regularly produces changes in the cells it infects profound enough to render their constituents auto-antigenic, as may be the case in some virus diseases characterized by acute necrosis of the infected cells (7).

When viewed in the large, the immunological reactions of the papilloma virus show a remarkable simplicity, and a striking similarity to

those of the classical antigens of immunology. It seems probable that the papilloma virus has a homogeneous antigenic structure, or at any rate, one less diverse than that of some of the larger viruses,—those which evoke complex immunological reactions, vaccinia (8), psittacosis (9), and myxomatosis (10), for example. Yet it possesses all the distinguishing properties of a virus, as Shope demonstrated (1).

Have the findings of the present work any implications as regards the tumor problem? In answer to this question it may be pointed out that the immunological study of a virus-induced tumor has led to the finding of a complement-binding antigen in extracts of the growth which is closely associated, if not identical, with the virus causing it. Whether similar substances are present in tumors of unknown cause is a matter now under investigation. It may be stated already that extracts of the transplantable Brown-Pearce tumor of rabbits also contain a complement-binding antigen which is similar, in some of its general traits, to that derived from the virus-induced papilloma (11). Later papers will report the findings of a detailed study.

SUMMARY

A study has been made of the yield of virus and of the complement-binding antigen from the virus-induced papillomas of cottontail and domestic rabbits. Extracts of the discrete, naturally occurring papillomas of cottontail rabbits usually contain virus in large amount; and, as a rule, they also contain the complement-binding antigen in high titer. The confluent growths produced experimentally with the virus in some cottontails, on the other hand, often fail to yield the virus, or furnish it in small amount; and extracts of them have little if any complement-binding capacity. The sera of cottontails with massive papillomas from which the virus cannot be recovered often have high antibody titers.

Many extracts were tested of the virus-induced papillomas of domestic rabbits. None contained the virus in large amount, and the majority of them failed to manifest it on sensitive test. A few fixed complement in low titer when mixed with immune sera, but most failed to do so. Crude extracts of the "non-infectious," virus-induced papillomas of domestic rabbits stimulated the formation of virus-neutralizing and complement-binding antibodies in low titer when

injected intraperitoneally into normal rabbits of the same breed, but Berkefeld filtrates of the same materials proved devoid of this immunizing effect.

The significance of the findings described in the three papers is discussed. The evidence as a whole favors the view that the virus stimulates the formation of the virus-neutralizing and complement-binding antibodies *in vivo*; and many facts indicate that it is closely associated, and in all probability identical, with the antigen that reacts with immune serum to fix complement *in vitro*.

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INTRAPERITONEAL AND INTRACEREBRAL ROUTES IN SERUM PROTECTION TESTS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

II. Mechanism Underlying the Difference in Protective Power by the Two Routes

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In the first paper of this series (1) it was shown that when mixtures of the virus of equine encephalomyelitis, Eastern or Western strain, and its antiserum are inoculated intraperitoneally in 12 to 15 day old mice, protection is obtained against much larger amounts of virus than when the mixtures are given intracerebrally. That is, with the same material, 1 to 1,000 cerebral infective doses of virus are neutralized by the intracerebral method as compared with 10,000 to 1,000,000 peritoneal doses by the intraperitoneal.

The purpose of the present study was to determine the mechanism underlying the more potent action exerted by the antiviral serum by one route than by another. At the outset it should be mentioned that it has not proved possible thus far to elucidate this completely; the experimental results have served to eliminate, however, divers theories hitherto offered to account for the phenomenon, to delimit more closely the place where the immune mechanism may be consummated and, what is more important, to correlate the mechanism of immunity with the pathways taken by the virus from the point of inoculation at various sites to the central nervous system.

In the first communication (1), mention was made of the work with other viruses in which it was shown that the protective power of serum-virus mixtures depends to a large extent on the route of inoculation. A summary follows of some of the interpretations of this reaction presented by earlier investigators, together with the bearing of our previous work on the question as it applies to equine encephalomyelitis in the mouse.

Thus Craigie and Tulloch (2) considered the possibility that differences in protection according to route might be explained, in the case of vaccine virus and its antiserum, on the ground of greater susceptibility of one organ (e.g., testis) to the virus than another (skin). On the other hand, Sabin (3) studied the problem of greater sensitivity of certain tissues to the action of vaccinia, herpes, B virus, and pseudorabies viruses, to note whether smaller amounts of these infective agents could be detected that way and hence more serum required for protection. With strictly quantitative methods in which the minimal infective doses were the same by two different routes, and by varying the amounts of serum and virus, Sabin found that the difference in protective capacity of mixtures could not be ascribed to the fact that one route may be more sensitive than another in detecting small amounts of virus. The results with equine encephalomyelitis virus, as already reported (1), confirmed the latter findings. With this virus, the lesser degree of protection by a given route (intracerebral) did not depend on the greater sensitivity of that tissue for detection of the infective agent. It was shown that one minimal infective dose of virus by the intraperitoneal route in 12 to 15 day old mice was approximately the same as by the intracerebral, yet the same amount of serum protected against many more doses by the former than by the latter route of inoculation.

Andrewes (4) obtained greater protection with antivaccinial serum by intradermal inoculation than by other routes and he suggested that this might be due to less ready diffusion of antibody in that site. Again the experiments of Sabin (3) indicated that this might not be the explanation: when the virus was used as a suspension of testicular tissue, marked diffusion took place from the action of the Duran-Reynals spreading factor of testicular tissue but the protective effect of the serum was, nevertheless, apparent.

That the difference in protective power depends on unknown factors peculiar to the tissue itself was indicated by the phenomenon described by Shope (5) in which mixtures of immune serum and pseudorabies virus which are innocuous subcutaneously in the guinea pig, produced fatal infection when given by the same route to the rabbit. This problem was also studied quantitatively by Sabin (3) and it was disclosed that the varying protective capacity of anti-pseudorabies serum in rabbits and guinea pigs was not due to the greater sensitivity of the subcutaneous tissue of rabbits in revealing smaller amounts of virus but rather to other conditions in this tissue of both species that may be only indirectly related to their susceptibility. Thus the results depended more on species involved than on route of inoculation. In this connection the investigator also studied the possibility of pseudorabies virus being fixed or entering the susceptible cells more rapidly or in greater quantity than the immune serum when the two are injected subcutaneously in rabbits. One must restrict oneself to measured quantities of materials in such trials: when such quantitative relationships were brought into consideration (3), and when subeffective amounts of serum were injected at intervals before virus in the same cutaneous sites, the writer concluded that no protection was gained. It is therefore probable that the poor protection by this route did not depend on the more rapid fixation of the virus by the dermal cells.

Findlay (6) investigated anew the finding of Francis and Magill (7) that antiserum of the virus of Rift Valley fever protected against more virus when the serum-virus mixtures were given mice intraperitoneally rather than intranasally. Findlay pointed out that in this instance the difference in protective capacity depended on the amount of inoculum, for when the dose was equal no variation occurred. In the preceding paper (1), it was shown, however, that with equine encephalomyelitis virus, variation in the protective capacity of antiserum by intraperitoneal and intracerebral methods of injections persisted when the amount of inoculum given was the same for both routes. In addition, the variation was still evident not only when the ages of the mice employed in the two methods were equal but also when the serum-virus mixtures were administered either freshly prepared or incubated for 2½ hours at 37°C.

It was brought out previously (1) and this is in agreement with the work of Sabin on other viruses (3), that the variation in protective power by the two routes, intracerebral and intraperitoneal, was in itself evidence that the action of the antiserum against equine encephalomyelitis virus was not an *in vitro* effect but was contingent upon the tissues into which it was injected.

The foregoing summary brings to light the fact that while the precise mechanism underlying the variation in the protective potency of antiserum-virus mixtures, when administered by different routes, is still unknown, certain theories advanced to explain this variation are not consistent with experimental data subsequently obtained. The virus now being studied was found to resemble in a general way several other viruses in that the effect of its antiserum is not consummated *in vitro* and that the variation in protective capacity is not primarily the result of the dose of inoculum or incubation of serum-virus mixtures but is influenced by the tissues into which such mixtures are injected (1). In this latter connection, no definite proof could be offered that the variation is dependent on the greater sensitivity of the tissues of one route to detect small amounts of virus over that of another.

For the purposes of the present investigation, namely, the elucidation of the mechanism involved in the variation of protective capacity by two routes of inoculation, the approach to the problem consisted of inoculation of serum intraperitoneally followed by the injection of virus by various routes. The results were then correlated with the pathways known to be taken by the virus according to the route of inoculation. In so doing, use was made of the knowledge at hand of the pathogenesis of the Eastern strain of the virus, that is, of the pathways pursued by it after its inoculation into mice at various sites.

Sabin (8) and Sabin and Olitsky (9, 10) have employed a method of partial serial tissue section of mouse (and guinea pig) central nervous system combined with tests for detection of virus by inoculation of animals, and have been able thereby to indicate the localization of lesions and virus, thus delineating the probable pathways of the infective agent from the periphery to the central nervous system. After intranasal instillation of Eastern equine encephalomyelitis virus into young or old mice, the central nervous system is invaded along the olfactory pathway. After intraperitoneal or intramuscular injection of this virus into 15 day old mice, it becomes demonstrable in the blood, and in the greater number of the animals, it migrates from the blood onto the nasal mucosa, whence it invades the central nervous system by the olfactory pathway. In some of these mice, however, invasion of the central nervous system occurs along the local peripheral nerves or along the auditory nerve pathway and possibly along the seventh nerve fibers. It has also been shown that while the virus enters and persists or multiplies in the circulating blood, no evidence was found of a direct passage of virus across the blood vessels of the brain (8–10).

Methods

These were essentially the same as those previously employed (1). The serum studied was the hyperimmune rabbit serum—the same sample was used throughout this work; the virus, the Eastern strain of equine encephalomyelitis, and the mice, the Rockefeller Institute albino strain of 12 to 15 days of age (unless otherwise mentioned). The mode of procedure, preparation of materials, and dosages were described in the first paper (1) and need not be repeated here.

Experiments on Passive Immunity

It was planned to inject mice with hyperimmune serum intraperitoneally in varying amounts and to follow this at certain intervals by administration of virus in different sites,—brain, nose, peritoneal cavity, and leg muscles. In other tests antiserum-virus mixtures were inoculated into the tissues mentioned; the results of the latter trials would serve as a check on those in which the serum was injected in advance of the virus. It was believed that by these means the virus could be placed either in the brain itself or in peripheral sites from which regions it would invade the central nervous system via the pathways already designated; the virus and tissues would then be under the influence of antiviral serum given either along with or before the infective agent. From the outcome of such experiments indications might be derived as to the relationship of pathways traversed to the effectiveness of serum neutralization.

Passive immunization experiments with this virus have already been reported. Howitt (11) introduced serum into the muscles of guinea pigs and found them resistant to intracerebral inoculation of virus; also Rottgardt and Riglos (12) gave serum intraperitoneally to guinea pigs and found them refractory either to an intracerebral or to a combined intranasal and subcutaneous test dose. These experiments were not performed on a quantitative basis, thus making it impossible or difficult to apply these data to the elucidation of the problem under consideration here.

In the following experiment an effort was made to determine quantitatively the protective capacity of a certain amount of serum, given in the peritoneal cavity, against virus introduced directly into the brain.

Serum Given Intraperitoneally, Virus Intracerebrally.[1]—In the first test 15 day old mice were injected intraperitoneally with the rabbit hyperimmune serum in the amounts indicated in Table I, and Eastern equine encephalomyelitis virus intracerebrally in varying doses and at the intervals as noted in the table in Experiments 1 to 3.

The results recorded indicate that even with as much as 2 cc. of hyperimmune serum given intraperitoneally—the largest amount that can be given safely in this way to 15 day old mice—either very little protection or only a questionable one could be secured against virus introduced intracerebrally 24 hours later. The result is similar to that achieved when serum-virus mixtures were used (1). There is striking contrast between this effect and that resulting from the inoculation of 1/133 of this amount (0.015 cc.) of serum mixed with virus and administered intraperitoneally whereby protection was afforded against 10,000 to 1,000,000 infective doses (1). From these tests it is apparent that a low degree of protection, or none at all, results when the virus is given into the brain which is the main seat of viral attack. The next step was to disclose any difference in reaction when the virus was inoculated peripherally rather than centrally; the nasal route was the first to be tried.

Serum Given Intraperitoneally, Virus Intranasally.—Sabin (13) has already shown that the intraperitoneal injection of 1 cc. of immune serum, or the equivalent of 50 cc. per kilo, given 4 hours before infection, protected mice against a lethal amount of this virus instilled

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[1] All such operations were performed with the aid of ether anesthesia.

TABLE I

Passive Immunity to Virus Given by Intracerebral, Intranasal, Intraperitoneal, and Intramuscular Routes

Experiment No.	Amount of serum intraperitoneally — Immune cc.	Normal cc.	Interval between serum and test with virus hrs.	Route of injection of virus	\(10^{-1}\)	\(10^{-2}\)	\(10^{-3}\)	\(10^{-4}\)	\(10^{-5}\)	\(10^{-6}\)	\(10^{-7}\)	\(10^{-8}\)	\(10^{-9}\)	Intracerebral doses	Intranasal doses	Intramuscular doses	Intraperitoneal doses
														Minimal infective doses in terms of route, against which there was protection			
1	0.05		24	ic	—	—	—	—	—	3	—			10+			
	2.0		24	"	—	—	3	—	—	3	3	3	—	10+			
			"	"	—	—	—	—	—	—	3	3	0	Control			
2	2.0		24	"	—	—	—	—	2*	3	3	3	—	10+			
				"	—	—	—	—	—	3	3	3	3	Control			
3	2.0		24	"	—	—	—	2*	2*	3	2+	—		1+			
			"	"	—	—	—	—	—	3	3	—		Control			
4	0.015	4	4	in	3	3	3	2	4†	3†	2†			1			
	0.015	4	4	"	3	3	3	0	—	3	3			Control			
	0.015	4	4	ic	—	—	—	—	—	—	—	—		0			
	0.015	4	4	"	—	—	—	—	—	—	—	—		Control	10,000 or more		
5	0.015	4	4	im	—	0	0	0	0	0	0	0				100,000 or more	
	0.015	4	4	"	0	0	0	2	3	3	3	0				Control	
	0.015	4	4	ip	0	—	—	0	—	0	—						
	0.015	4		"	0	0	0	3	3	3	0						

Dose of virus dilution by any route was the same, namely, 0.03 cc.
ip, indicates intraperitoneal; ic, intracerebral; in, intranasal; im, intramuscular; —, no test made.
+ Undetermined but equal to or less than the amount indicated.
* One died of the inoculation of serum.
† Four mice inoculated.

intranasally. It was now desirable to know whether the small amount of serum used in our experiments for demonstration of protective antibody by the intraperitoneal route (0.015 cc.) might have the same or a different effect. As a control, and furthermore to check the results obtained in the previous passive protection test in which the intracerebral route for inoculation of virus and large amounts of serum were used, this latter test was repeated with the small amount of antiserum.

For this purpose, 15 day old mice received rabbit serum intraperitoneally—one group, normal serum and the other, hyperimmune—in a dose of 0.015 cc. each. The sera were diluted 1:1 in saline solution so as to make for greater accuracy in measurement of the amounts needed as inocula. 4 hours later they were given either intranasal or intracerebral test doses of various dilutions of virus, the dose by both routes being 0.03 cc. The results are shown in Experiment 4, Table I.

The data reveal that in the mice receiving virus peripherally, no protective influence of the serum could be discerned. As was to be expected, the control group of animals having been given virus centrally, again showed a low degree of protection, that is, against only one infective unit. A similar outcome was found on repetition, as recorded in Table II. It would appear, therefore, that by intranasal and intracerebral methods the immune serum exhibited in both instances the same low or ineffective neutralization.

The results are now reported of passive immunity tests in which virus was given by other peripheral routes, namely, the intramuscular and intraperitoneal.

Serum Given Intraperitoneally, Virus Intraperitoneally or Intramuscularly.—The plan of this experiment was to introduce immune serum intraperitoneally and to follow it later by the simultaneous injection of virus, intramuscularly in one group of 15 day old mice and intraperitoneally in another.

As will be noted in Experiment 5, Table I, hyperimmune rabbit serum was given intraperitoneally in doses of 0.015 cc. (as in Experiment 4) followed 4 hours later by an intramuscular inoculation (muscles of a posterior extremity) of 0.03 cc. of each dilution of virus. (A preliminary titration of virus activity by this route exhibited the limiting titer of infectivity in the 10^{-3} dilution.) Intraperitoneal inoculations were made at the same time in another group of serum-treated 15 day old mice, employing the same amount of inoculum, 0.03 cc.

The results reveal that the immunity to intramuscularly or intra-peritoneally injected virus after serum has been given intraperitoneally is of a remarkably high degree. The serum induced protection against at least 10,000 minimal intramuscular infective doses introduced into the muscles, and at least 100,000 peritoneal units inoculated into the peritoneal cavity. (A still higher degree of resistance to intramus-cularly introduced virus is shown in Table II.) It is therefore clear that on the basis of effective protection as revealed by the methods employed, the administrations of virus by the intramuscular and

TABLE II

Passive Immunity to Virus Introduced Intracerebrally, Intranasally, and Intramuscularly

Amount of serum Immune cc.	Normal cc.	Interval between serum and test with virus hrs.	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which there was protection		
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intracerebral doses	Intranasal doses	Intramuscular doses
0.015		5*	—	—	—	—	3	3	1	0	0			
	0.015	5*							3	1		1 Control		
0.015		4½*			3	3	0	0	0	1	0			
	0.015	4½*			3	2	2	1	1	0	0		1 Control	
0.015		4	1	0	0	0								
	0.015	4						3	3	2	0			10,000,000 Control

Abbreviations as in Table I.
Mice were a mixture of the ages of 12, 13, and 14 days.
* Intervals due to the time used in inoculation.

intraperitoneal routes (peripheral) align themselves in one class of high protective capacity, the nasal (peripheral) and the cerebral (central) in another of low protective power. However, all the experiments on which this classification is based were not performed at the same time; this factor was therefore taken into consideration in the next test in which was studied the simultaneous use of intra-cerebral, intranasal, and intramuscular methods.

Comparison of Results with Serum Given Intraperitoneally and Virus Intracerebrally, Intranasally, and Intramuscularly.—The procedures followed in this experiment are outlined in Table II. From the

results it is apparent that again hyperimmune serum injected intraperitoneally yielded protection against only one cerebral or nasal infective unit when virus was administered intracerebrally or intranasally, and against as many as 10,000,000 intramuscular units when the virus was inoculated intramuscularly. The remarkable fact brought to light is that such a relatively minute quantity of antiserum (0.015 cc.) can be capable of exerting so high a degree of protective effect, even though the serum is introduced into the abdominal cavity and the virus into the muscle. Another interesting observation is revealed in these experiments as well as in those recorded in Table III. Less virus is required to induce encephalitis after its intramuscular rather than intranasal introduction. This may be taken as evidence that the virus may multiply before arriving at the central nervous system. The larger amounts of virus needed to produce encephalitis after its intranasal instillation may be explained by the fact that a great deal of it is washed away. It should be stressed also that the intramuscular minimal infective dose of virus is the same as the intracerebral and often the same as the intraperitoneal.

Up to this point experiments were made on the basis of passive immunity with the antiserum given prior to the virus. The results, however, are in accord with those already reported (1), which were derived from tests with serum-virus mixtures injected intracerebrally and intraperitoneally. In the following a comparison was made of the effects secured from the inoculation of serum-virus mixtures intranasally as an example of a route by which only low protective potency is demonstrable, and intramuscularly, where high protective capacity is discerned.

Comparison of Infectivity of Serum-Virus Mixtures by Intranasal and Intramuscular Routes

Serum-virus mixtures, without incubation, were inoculated intramuscularly or intranasally into groups of mice 14 or 15 days old. The amount of inoculum by both routes was 0.03 cc. prepared as previously described (1) and contained 0.015 cc. of serum. The results are given in Table III.

The outcome of this experiment is plain: the antiserum in the mixtures protected against 10 infective nasal units of virus by the intranasal, as against 1,000,000 intramuscular doses by the intramuscular route. There is very little difference in effect when immune

serum is mixed with virus just before animal inoculation or when the serum is given separately and prior to the virus. Here again occurs the wide variation in protective capacity elicited by two routes of inoculation.

The data can be related to the pathways traversed by the virus after inoculation by these various routes. With the intranasal and intracerebral methods, the virus enters nervous tissue immediately since in the former it progresses along the olfactory pathway to the brain, and in the latter it is placed within the brain itself, the cells of which it attacks directly. But after inoculation into the peritoneal cavity or into the muscles, virus circulates in the blood and is demon-

TABLE III

Comparison of Infectivity by Intranasal and Intramuscular Routes

Route of inoculation of serum-virus mixtures	Serum	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which there was protection	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intranasal doses	Intramuscular doses
in	HR	—	1	3	0	0	—	—	—	—		
"	NR	—	0	3	3	2	—	—	—	—		
im	HR	1	—	0	3			—	—	—	10	
"	NR	—	—	0				3	1	0	Control	1,000,000
												Control

Abbreviations as in Table I.
HR, hyperimmune rabbit serum; NR, normal rabbit serum.

strable there (9, 10). Thus it appears that the large amounts of protection may in some way be related to an action occurring at the peripheral site or during the passage of the virus through the blood on its way to the central nervous system. Attention was then turned to a study of the relation of the circulating blood to the mechanism of the immune reaction.

Virus in Blood Following Intraperitoneal Injections of Serum-Virus Mixtures

It has been shown (9, 10) that virus may be detected in the blood of young mice inoculated intraperitoneally or intramuscularly. In a preliminary test it was found that virus could be recovered from

the blood at 1, 2, 4, and 24 hours (longer periods of time were not studied) after intraperitoneal introduction of virus, which is in general agreement with the prior observations. The plan of the following tests was to inject serum-virus mixtures by this route and to observe whether virus could be detected later in the blood stream.

Mixtures of 10^{-3} dilution of virus (1,000 cerebral infective units) and hyperimmune rabbit serum were injected intraperitoneally (dose 0.03 cc.) in a group of 14 day old mice. At the same time 10^{-6}, 10^{-7}, and 10^{-8} dilutions of virus mixed with normal serum were similarly inoculated into another group. 24 hours later subgroups of each were bled to death by cardiac puncture by means of syringes washed with sterile 1:500 solution of heparin. The blood secured was directly transferred to other mice intracerebrally (dose 0.03 cc.). The remainder of the animals which were not bled were observed for signs of infection with the virus, as controls. The results are summarized in Table IV.

It is clear from Table IV that by the methods used, virus was detected in the blood 24 hours after intraperitoneal injection of normal serum-virus mixtures but not in the animals receiving the immune serum.

The work of Smith (14), Long and Olitsky (15), and Sabin (16) has shown that after intravenous injection of vaccine virus into normal rabbits, the virus can be demonstrated in the whole blood. However, following similar injections of immune serum-virus mixtures, or of virus into immune rabbits, the infective agent can be recovered from leucocytes but not from whole blood. An attempt was therefore made to search for virus in the leucocyte suspensions in mice given normal and immune serum-virus mixtures intraperitoneally.

Groups of mice were inoculated intraperitoneally with normal and immune serum-virus mixtures as in the last experiment. The same samples of serum were used; dilution of virus was 10^{-3}; dose, 0.03 cc. Mice were 14 and 15 days old. 24 hours later they were bled from the heart as before and the heparinized blood from each of the two groups of animals was pooled. The washed buffy coat of the centrifuged blood was secured and injections of these leucocyte-containing suspensions were made into adult mice in groups of six. The result was that all the mice receiving leucocyte suspensions derived from mice given normal serum-virus mixtures died and all those injected with immune-serum preparations survived.

By means of the methods here employed virus was found to be present constantly in the whole blood or in its leucocyte layer after

VIRUS OF EQUINE ENCEPHALOMYELITIS. II

normal serum-virus mixtures were given intraperitoneally. By the same methods, no virus was apparently detectable in these materials

TABLE IV

Test for the Presence of Virus in the Whole Blood of Mice 24 Hours after Intraperitoneal Inoculation with Normal and Immune-Serum-Virus Mixtures, Respectively

Mouse No.	Dilution of virus in mixture given intraperitoneally	Serum	Fate of ones not bled	Results of test for virus in the whole blood*
1	10^{-3}			
2	10^{-3}	HR		
3	10^{-3}	"		0/3
4	10^{-3}	"		0/3
5	10^{-3}	"		0/3
6	10^{-3}	"	S	†
7	10^{-3}	"	"	
8	10^{-3}	NR		
9	10^{-3}	"		3/3
10	10^{-3}	"		3/3
11	10^{-3}	"	E	3/3
12	10^{-3}	"	"	
13	10^{-6}	"	"	
14	10^{-6}	"		
15	10^{-6}	"	"	
16	10^{-7}	"	"	
17	10^{-7}	"	"	
18	10^{-7}	"	"	
19	10^{-8}	"	"	
20	10^{-8}	"	"	
21	10^{-8}	"	S	

S, survived; E, encephalitis and death.
Other abbreviations as in Tables I and III.
* Numerator indicates the number of mice developing encephalitis; denominator indicates the number injected.
† No blood obtained.

after immune serum-virus preparations were similarly introduced into mice.

In Vitro Effect of Normal Mouse Blood on Serum-Virus Mixtures.—While these tests for virus in the blood could not be considered conclusive, they were taken as evidence that in some manner the immune

serum prevents the circulation of virus in the blood.   The precise mechanism by which this is done is not apparent.   Although it is known that serum does not have an inactivating effect in the test tube, there was a possibility that the whole blood of the normal animal might enhance the action of antiviral bodies.   The following experiment was performed to test this point.

Hyperimmune rabbit serum plus normal heparinized blood obtained by pooling whole blood from 18 normal, 15 day old mice, plus dilutions of virus in broth, were mixed, using 0.3 cc. of each ingredient.   The final dilutions of virus were 10^{-1} to 10^{-9}.

TABLE V

Effect of Heparinized Blood on the Protective Power of Serum When Mixtures of Blood, Serum, and Virus Are Injected Intracerebrally

Blood, or broth control	Serum	Number of mice developing encephalitis of three injected									Minimal cerebral infective doses of virus against which the serum protected with	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Blood	Broth
Blood	HR	3	3	3	3	3	—	—	—	—	100*	
	NR	—	—	—	—	—	3	3	3	0	Control	
Broth (control)	HR	—	—	3	3	2	0	—	—	—		1,000
	NR	—	—	—	—	—	—	3	3	2		Control

Abbreviations as in Table I.
* Undetermined but equal to or less than the amount indicated.

Another mixture was prepared in precisely the same way except that normal rabbit serum was substituted for the immune serum; other controls are also shown in Table V.   Of each of these materials, 0.03 cc. was withdrawn and injected intracerebrally in mice.   These components were in contact in the test tube during the period of animal inoculations, which contact lasted from about 10 minutes before the first injection to about 30 before the last.

It will be noted from the table that normal mouse blood *in vitro* probably contained no factor enhancing the protective power of immune serum, nor was the immune reaction found to be completed therein.   It is natural to conclude from all these tests with blood that under the influence of immune serum after intraperitoneal injection of serum-virus mixtures, virus is not found in the blood.

Résumé

In the first paper of this series (1) it was shown that antiserum against equine encephalomyelitis virus protected against many more minimal infective doses when serum-virus mixtures were given intraperitoneally, instead of intracerebrally. It was indicated that this variation in protective capacity by two routes of inoculation (*a*) was not due to inactivation of virus by serum *in vitro*, (*b*) that it did not depend on the greater sensitivity of tissues to detect virus, nor (*c*) on the amount of inoculum, nor (*d*) on incubation of the mixtures.

The purpose of the present investigation was to throw more light on the mechanism underlying the phenomenon. Our procedure consisted of passive immunization of 12 to 15 day old mice by intraperitoneal inoculation of hyperimmune serum followed by virus given in the brain, nose, muscles or peritoneal cavity. When intracerebral or intranasal inoculations of virus were given, only minimal protection or none at all was demonstrated; when intramuscular or intraperitoneal injections were made, a marked protection was revealed. Further experiments exhibited an agreement in effect when antiserum and virus were mixed and then without incubation were introduced into animals. The results bring out the fact that a relatively minute amount of antiserum (0.015 cc.) has the capacity to protect against a remarkably high amount of virus given intramuscularly or intraperitoneally, even against as many as 1,000,000 to 10,000,000 infective doses.

The experimental findings on the variation in the protective capacity by different routes of inoculation appear to be correlated with the pathways traversed by the virus from the periphery to the central nervous system. Since after intraperitoneal or intramuscular inoculation the pathway includes the circulating blood, studies were made on the blood. After introduction of serum-virus mixtures intraperitoneally, virus was not found in the circulation 24 hours later.

DISCUSSION

An interpretation of the results will be proposed on the basis of existing knowledge as to the pathways traversed by the virus of equine encephalomyelitis after inoculation by various routes in young mice.

It is known (8) that virus given intranasally in mice reaches the bra by the olfactory chain of neurons and this pathway is thus entire within nervous tissue. Also, virus placed within the brain com into direct contact with the nerve cells. After intramuscular a intraperitoneal inoculation, however, virus, in the greater number mice, reaches the central nervous system from the periphery throu the mediation of the blood stream. That is, the infective agent deposited from the blood onto the nasal mucosa whence the invasi of the brain is by way of the olfactory pathway (9, 10). The expe ments have shown that when the pathway of virus is only in nervo tissue there occurs little or no protection by antiserum; when t pathway includes the circulating blood, on the other hand, the pr tective capacity is great, even though relatively minute amounts antiserum are used.

Interest centers on the point along the pathways taken by t virus where the immune effect is consummated. It is not likely be in the brain because after the intraperitoneal injection of antiser there is often neutralization of only one minimal intracerebral infecti dose of virus or even none at all. Because of the fact that after int muscular or intraperitoneal injection of virus it migrates in m cases from the blood stream to the nasal mucosa, there was the p sibility that the immune reaction took place on the nasal muco However, it was found that when serum is given intraperitonea and followed by virus intranasally, little or no protection occurred, that this possibility seemed unlikely. By elimination, then, the m important part of the immune reaction is probably effected son where in non-nervous tissue. But the experiments leave undete mined the exact tissue or site of the reaction.

In the latter connection, antiserum injected intraperitoneally f lowed by virus intramuscularly results in a high degree of prote tion. If the action of the serum is to protect the muscle cells at t local site of inoculation, antibody must have entered the blood strea before reaching the muscle. Plainly, great diffusion is possible the blood and therefore the suggestion alluded to earlier that an vaccinal serum is more effective in one tissue than in another becau of less diffusion (4) may not apply here. Although by the metho used virus was not shown to be present in the blood 24 hours af

intraperitoneal inoculation of serum-virus mixtures, no conclusive proof is adduced from this fact and from the *in vitro* experiments as reported, that the reaction takes place in the blood stream. Sabin (13) had previously demonstrated that with the virus now under investigation the prior nasal instillation of antiserum produces resistance against infection by the same route, 2 to 4 hours later, provided the dose of virus is not too great, by virtue of the local specific protective action on the cells by the serum.

A corollary may be offered to strengthen the general proposition that the protective capacity varies, depending on whether nervous or non-nervous tissues are included in the pathways of virus progression to the central nervous system from the point of inoculation. Sabin (3, 13, 17, 18) has shown that, following injection of pseudorabies (or B virus) intramuscularly, the virus multiplies locally and then progresses to the central nervous system by way of the peripheral nerves; furthermore, injection of serum-virus mixtures intramuscularly results in protection, while intracerebrally there is none. It is possible that the difference may be ascribed (18) to the fact that the antiserum prevents the local multiplication of virus and hence no progression along peripheral nerves occurs. Pseudorabies antiserum-virus mixtures are infective intracerebrally even when minimal doses of virus are used, but do prevent infection when administered by the intranasal (or subcutaneous) route. The question is whether the antiserum here acts in the same way to prevent local multiplication of virus and so no further progression can take place. By way of contrast, pseudorabies virus does not take the olfactory pathway as equine encephalomyelitis does (8), therefore the intranasal route is one by which pseudorabies virus progresses first through nonnervous tissue; consequently one should expect a higher protective capacity by this route than by the intracerebral.

One final point, perhaps of practical bearing, remains for discussion,—if one assumes that in the horse as in the mouse immunity which results from circulating antibody depends similarly on the route of inoculation of virus. The most probable hypothesis as to the mode of transmission in nature of equine encephalomyelitis is that the virus is carried by mosquitoes. If this is so, the portal of entry is such in natural infection as to correspond with intraperitoneal

or intramuscular inoculation in the mouse, and the immunity from circulating antibodies might be expected to be maximal. Such a state of affairs would explain the effective use of antiserum in the field as a prophylactic agent (19, 20).

CONCLUSIONS

Minute amounts of antiserum injected intraperitoneally protect against large doses of equine encephalomyelitis virus given intramuscularly or intraperitoneally in 12 to 15 day old mice. Antiserum given intraperitoneally with virus intracerebrally or intranasally results in little or no protection. These phenomena occur as well when serum-virus mixtures are injected at the different sites. The marked variation of the protective capacity of antiserum as thus displayed would appear to be dependent upon the differing pathways of progression of the virus from the site of injection to the central nervous system.

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INTRAPERITONEAL AND INTRACEREBRAL ROUTES IN SERUM PROTECTION TESTS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

III. Comparison of Antiviral Serum Constituents from Guinea Pigs Immunized with Active or Formolized Inactive Virus

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It has been reported (1, 2) that the protective power of antisera against the virus of equine encephalomyelitis is much greater if serum-virus mixtures are inoculated into 12 to 15 day old mice by the intraperitoneal route, instead of by the intracerebral. The sera tested in the series of experiments referred to were from animals immunized by injections of active virus, or were derived from horses infected naturally.

During the course of these investigations sera obtained from guinea pigs which had been immunized with formolized, inactive virus (3) were submitted to similar comparative intraperitoneal and intracerebral tests for protective potency. The results were unexpected in that they differed from those obtained with sera from animals inoculated with active virus. This was considered important to the problem of the correlation of antibody to immunity and led to further study of the phenomenon. The outcome of this study forms the substance of the present communication.

It may be recalled that previous work (3) demonstrated that a high degree of resistance to experimental equine encephalomyelitis (against 1,000 intracerebral lethal doses of virus) can be induced in guinea pigs by the use of formolized vaccines in which no active virus can be detected. By the application of quantitative methods it was determined that it is necessary to introduce subcutaneously 3,000 to 30,000 mouse intracerebral infective doses of virus three times, at intervals of 7 days, to secure the same degree of protection in guinea pigs by the use of untreated active virus. Thus small amounts of active virus, detectable by animal inocula-

779

tion, are not sufficient to bring about immunity, while formolized vaccines in which virus is not demonstrable even by elaborate and extensive tests (3) will induce a high degree of resistance.

A continuation of these quantitative studies (4) revealed that guinea pigs immunized either with untreated, active virus or formolized, inactive virus, show no distinctive differences in the antiviral body content of their sera as determined by the mouse intracerebral test. In either event, although guinea pigs are found to resist 1,000 or more intracerebral doses of virus, the antibody content of their sera is, by this test, low and in some instances even absent.

Methods

The method of preparing formolized vaccine used in the experiments was that already described (3). The vaccines were made up of fresh mouse brains infected with the Eastern strain. A concentration of 0.5 per cent formalin was used throughout. Vaccines were kept in the dark at room temperature for 24 hours after the addition of formalin, and thereafter in the refrigerator at 5°C.

The other procedures—animal inoculation, neutralization tests, dosages, etc.— were fully described in the first two papers of this series (1, 2). In Table I will be found the details of immunization of guinea pigs with formolized vaccines and tests for immunity. The sera were obtained by bleeding from the heart[1] at the intervals noted, and this was always performed before the test dose for resistance was given. Equal parts of serum from two to four animals of each group were pooled.

EXPERIMENTAL

The first step in this investigation was the preparation of antisera by immunization of guinea pigs with formolized vaccines. Table I summarizes the results obtained.

The tabulated results indicate that of five groups of guinea pigs one received massive doses of formolized vaccine, i.c., ten times mor[e] than the largest amount used in any of the others. The interva[l] between the preparation of the vaccine and its use was from 35 t[o] 100 days,[2] between the last inoculation and the collection of seru[m] was from 14 to 16 days, and between the last inoculation and t[he] later intracerebral test for resistance was about 16 days (in the fi[rst] group this test was unsatisfactory, hence the repetition). In agr[eement]

[1] All operative procedures on animals were done with the aid of ether anesth[esia.]
[2] In a prior report (3) it was shown that the vaccines retained their immun[izing] property for at least 65 days; since then it has been demonstrated after 100 [days of] [sto]rage at about 5°C.

ment with previous experience (3) the vaccines used did not contain any active virus that could be demonstrated by the methods employed. Knowing that small, measurable amounts of active virus are not enough to induce immunity (3) it was believed that these guinea pigs could not have had immunity attributable to any but inactivated

TABLE I

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Guinea pig serum (see Table II)	Formolized vaccine			Immunization			Intracerebral test for immunity	
	Vaccine No.	Mouse infective units per cc. before inactivation	Test for active virus*	Interval between preparation and use	Route and dose	Interval between last injection and bleeding for serum	Minimal infective doses injected	Result†
				days		days		
1	X H	3×10^9	Negative	35	1 sc, 1 cc.‡	14, 16 (pooled)	—	—
3	"	3×10^9	"	100	1 sc, 1 cc.	14	1,000	0/2
4	"	3×10^9	"	100	2 sc, 1 cc. each, 7 d. int.‡	14	1,000	0/2
5	"	3×10^9	"	100	3 sc, 1 cc. each, 7 d. int.	14	1,000	0/1
6	XIII H	3×10^9	"	56	3 sc, 10 cc. each, 5–6 d. int.	14	1,000	0/2

* Test for virus included 15 day old mice injected intracerebrally (0.03 cc.) and intraperitoneally (1.0 to 2.0 cc.); eight other vaccines prepared for other purposes in exactly the same way have given negative tests for active virus.

† Figures exclude animals that died from the operations of bleeding from the heart or intracerebral inoculation. The numerator represents the number dead of encephalitis, the denominator, the number of animals given the test dose.

‡ 2 sc, 1 cc. each, 7 d. int. = two subcutaneous injections, 1 cc. each at 7 day interval.

virus. This point is stressed because a comparison is being made between the results of immunization by means of active and of inactive virus. Excluding the animals that died accidentally during bleeding or inoculation, all of those injected with vaccines were found to be resistant to 1,000 guinea pig intracerebral lethal doses of virus.

tion, are not sufficient to bring about immunity, while formolized vaccines in which virus is not demonstrable even by elaborate and extensive tests (3) will induce a high degree of resistance.

A continuation of these quantitative studies (4) revealed that guinea pigs immunized either with untreated, active virus or formolized, inactive virus, show no distinctive differences in the antiviral body content of their sera as determined by the mouse intracerebral test. In either event, although guinea pigs are found to resist 1,000 or more intracerebral doses of virus, the antibody content of their sera is, by this test, low and in some instances even absent.

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				days		*days*		
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active virus. Excluding the animals that died accidentally during
bleeding or inoculation, all of those injected with vaccines were found
to be resistant to 1,000 guinea pig intracerebral lethal doses of virus

TABLE II

Sera of Guinea Pigs Immunized with Formolized Vaccine.   Comparative Protective Power When Serum-Virus Mixtures Are Inoculated by Intraperitoneal and Intracerebral Routes

Experiment No.	Route of injection	Dose	Age of mice	Guinea pig serum (see Table I)	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intraperitoneal doses	Intracerebral doses
		cc.	days												
1	ip	0.1	14–15	1	3	1	3	2	2	0	0	—	—	10	
	"	0.1	14–15	2* (active virus)	2	0	0	0	0	0	0	—	—	100,000*	
	"	0.1	14–15	Normal	—	—	—	—	3	3	2	1	—	Control	
	ic	0.03	25±	1	—	—	—	3	3	3	1	0	—		10
	"	0.03	25±	2* (active virus)	—	—	—	3	3	0	0	0	—		100*
	"	0.03	25±	Normal	—	—	—	—	—	3	2	2	1		Control
2	ip	0.1	15	1	—	—	0	2	2	0	—	—	—	10	
	"	0.1	15	2* (active virus)	3	3	0	—	—	—	—	—	—	10,000*	
	"	0.1	15	Normal	—	—	—	—	3	3	2	0	—	Control	
	ic	0.03	25±	1	—	—	—	—	3	3	1	2	—		0
	"	0.03	25±	2* (active virus)	—	—	—	3	3	1	—	—	—		100*
	"	0.03	25±	Normal	—	—	—	—	—	3	2	2	1		Control
3	ip	0.1	12, 13, 14	3	—	—	1	3	1	3	—	—	—	1†	
	"	0.1	12, 13, 14	4	—	0	1	2	0	—	—	—	—	100	
	"	0.1	12, 13, 14	5	3	2	2	3	—	—	—	—	—	100†	
	"	0.1	12, 13, 14	Normal	—	—	—	—	3	2	1	—		Control	
	ic	0.03	26	3	—	—	—	—	3	3	3	0	—		1
	"	0.03	26	4	—	—	—	—	3	2	0	0	—		10
	"	0.03	26	5	—	—	—	—	3	2	1	0	—		10
	"	0.03	26	Normal	—	—	—	—	—	3	2	1			Control

ip, intraperitoneal; ic, intracerebral.

* Serum 2 was from a guinea pig that survived an immunizing intramuscular injection of 10^5 mouse infective units of virus and was bled 14, 17, and 25 days later, and the samples so collected were pooled.

† Undetermined but equal to or less than the amount indicated.

TABLE II—*Concluded*

Experiment No.	Route of injection	Dose	Age of mice	Guinea pig serum (see Table I)	Number of mice developing encephalitis of three injected									Minimal infective doses in terms of route, against which the serum protected	
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Intra-peritoneal doses	Intra-cerebral doses
		cc.	*days*												
4	ip	0.03	12	5	—	3	1	3	0	0	0	0	—	1,000	
	"	0.03	12	Normal	—	—	—	—	—	3	3	2	—	Control	
	ic	0.03	30±	5	—	—	—	—	3	1	0	0	—		10
	"	0.03	30±	Normal	—	—	—	—	—	3	1	0			Control
5	ip	0.1	12	4	—	2	1	2	0	0	—	—	—	1,000	
	"	0.1	12	Normal	—	—	—	—	—	3	3	2	—	Control	
	ic	0.03	Adult	4	—	—	—	3	2	2	0	0	—		1
	"	0.03	"	Normal	—	—	—	—	—	3	3	1	0		Control
6	ip	0.1	12	6	3	3	2	3	0‡	1	1	—	—	1,000	
	"	0.1	12	Normal	—	—	—	—	—	3	3	2	3	Control	
	ic	0.03	Adult	6	—	—	—	3	1	0	1	0	0		100
	"	0.03	"	Normal	—	—	—	—	—	3	2	0	0		Control

‡ One died of an accidental cause.

The serum employed in the present experiments could therefore be considered as being derived from guinea pigs highly resistant to artificial infection.

Comparative Intracerebral and Intraperitoneal Inoculations of Serum-Virus Mixtures.—The object of the following tests was to determine whether the same wide variation in protective capacity which follows the inoculation of serum derived from animals immunized with active virus would obtain with serum collected from guinea pigs immunized with inactive formolized virus. The data of the experiments are given in Table II and it is to be noted that in Experiments 1 and 2, as a control, a serum (No. 2) was included which was obtained from a guinea pig immunized with active virus.

From Table II it is evident, first, that the sera of guinea pigs immunized with formolized vaccines protected against only small numbers of minimal infective doses of virus when serum-virus mixtures were introduced intracerebrally in mice, which is in agreement with earlier observations (4); second, that the wide variation observed

previously by two routes of inoculation with sera of animals immunized with active virus did not obtain under present circumstances. For, when the serum and virus were tested by injection intraperitoneally in 12 to 15 day old mice, only a small difference was revealed

TABLE III

Sera of Animals Immunized with Formolized Vaccine or Active Virus

Serum No.*	Animal	Immunization	Minimal infective doses in terms of route, against which the serum protected	
			Intraperitoneal doses	Intracerebral doses
6	Guinea pig	Formolized vaccine	1,000	100
5	" "	" "	1,000	10
4	" "	" "	100	10
5	" "	" "	<100	10
1	" "	" "	10	10
4	" "	" "	1,000	1
3	" "	" "	<1	1
1	" "	" "	10	0
	Mouse	Active virus	1,000,000	1,000
	Rabbit	" "	100,000	100
2	Guinea pig	" "	100,000	100
2	" "	" "	10,000	100
	" "	" "	10,000	100
	Rabbit	" "	100,000	10
0814	Horse	Natural infection	100,000	10
	Rabbit	Active virus†	10,000	10
1	Horse	Natural infection	100,000	1
5	"	" "	100	1

Only those experiments have been included in which control was done with normal serum of the same animal species.

* Numbers of sera in vaccine series taken from Table II; those of active virus series, from Paper I (1), except serum 2, from Table II.

† Western strain.

between the number of minimal infective doses neutralized in this way and the number neutralized intracerebrally. Serum 2, from an animal immunized with active virus, did, on the other hand, reveal the variation, as was to be expected. For example, this latter serum showed 100 cerebral infective units neutralized intracerebrally and

from 10,000 to 100,000 peritoneal units intraperitoneally; whereas the vaccine serum showed 0 to 100 cerebral doses rendered inactive by intracerebral test and from 0 or 1 to 1,000 peritoneal infective units, intraperitoneally.

Comparison of Sera from Animals Immunized with Vaccine or Active Virus.—The next step was to summarize the results of all the experiments with the sera of guinea pigs immunized by means of inactive formolized virus, and of those animals treated with active virus, so as to determine the frequency and regularity with which this different reaction occurred.   Table III gives this summary.

It can be seen that sera of guinea pigs immunized with formolized vaccine and neutralizing 100 cerebral doses intracerebrally, protect against 1,000 peritoneal doses intraperitoneally.   However, sera of animals immunized with active virus exhibiting the same degree of neutralization intracerebrally, protect against 10,000 or 100,000 doses intraperitoneally.   Also, vaccine sera protecting against 10 doses by the cerebral route, neutralize 10 to 1,000 by the peritoneal, while active virus sera giving this same amount of protection cerebrally, inactivate 10,000 to 100,000 peritoneally.

The results, based on average counts, are as follows: The average number of intraperitoneal infective units neutralized by the formolized virus sera was 400 and in the case of active virus sera was about 153,000.   This is a striking difference.   The average number of intracerebral infective units protected against by the formolized virus sera was about 17 and in the instance of active virus sera was approximately 140.

Even when the amount of vaccine was increased 10 times that ordinarily employed to render guinea pigs firmly resistant, such variation in serum protective capacity by the two routes, as is seen when untreated virus is given as immunizing agent, was not encountered.

DISCUSSION

Guinea pigs immunized either with active virus or with formolized inactive virus have a high degree of resistance to virus injected intracerebrally.   While the sera derived from guinea pigs immunized with active virus and those with formolized, inactive virus show the same range of low protective capacity when serum-virus mixtures

are inoculated intracerebrally in mice, a striking difference is revealed when the two sera are injected intraperitoneally. Then serum of animals rendered immune by means of active virus exhibits high protection and that collected from guinea pigs immunized by formolized vaccines reveals only low protective power, approaching the neutralization titers obtained by the intracerebral method. The importance of this phenomenon centers chiefly on the possibility of two antibodies being involved in the action of the two sera.

With respect to the nature of the antibody, the following assumptions may be made.

1. The antiviral body in both sera is a single antibody, the different results obtained by the intracerebral and intraperitoneal methods of testing depending on the quantities of it that are present. This is unlikely, however, although both sera protect against the same low amounts of virus, or do not protect at all, when tested intracerebrally, they are not of equal value in their neutralizing power when injected intraperitoneally.

2. There are two antibodies present; that is, in the serum of guinea pigs immunized with active virus the antibody has properties different in effect from that of animals receiving injections of formolized, inactive virus. (a) The difference may be ascribed to the fact that when active virus is employed as immunizing agent, infection is induced and multiplication of virus occurs; when inactive virus is given, no infection or multiplication takes place. The antigenic stimuli in both instances may evoke different antibodies, detectable by intraperitoneal test. (b) There is a further suggestion that the "intracerebral" antibody may be present in both sera and the "intraperitoneal" one in greater concentration in serum derived from animals immunized with active virus and to a much lesser extent in that from guinea pigs injected with formolized vaccines. In other words, the reactions of the sera may be conditioned by varying amounts of these substances present.

3. Finally, neither of these possibilities may apply satisfactorily, and the solution of the problem remains still obscure. The present results, however, lend more support to the supposition of the existence and the operation of at least two antibodies, irrespective of their quantitative distribution in the two kinds of sera.

There is still another consideration.   Although the protective capacity of sera secured from guinea pigs immunized by means of formolized, inactive virus is low when tested by intraperitoneal inoculation, nevertheless such animals have a high degree of resistance to virus injected intracerebrally.   This is consistent with the hypothesis that the content of antiviral antibody is not proportional to the degree of resistance to infection (5).

SUMMARY

Earlier experiments had shown that the sera of animals immunized with active virus have much greater protective potency when serum-virus mixtures are injected intraperitoneally into 12 to 15 day old mice than when given intracerebrally.   The present work was concerned with similar tests on sera derived from guinea pigs immunized by vaccines in which the virus had been inactivated by formalin.

In comparing the content of antiviral body by means of intracerebral and by intraperitoneal inoculation, it was found that both sera show about the same low degree of neutralizing capacity by the former method.   By intraperitoneal inoculation, on the other hand, serum collected from guinea pigs immunized by means of active virus reveals high protective power, while that from animals receiving formolized, inactive virus exhibits lower neutralization titers which approach those obtained by the intracerebral method.   The significance of this unexpected finding is discussed.

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STUDIES ON THE NASAL HISTOLOGY OF EPIDEMIC INFLUENZA VIRUS INFECTION IN THE FERRET

I. The Development and Repair of the Nasal Lesion

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PLATES 36 TO 39

During the past few years a considerable body of evidence has accumulated which suggests that immunity to influenza virus infection in man, in swine, and in ferrets is closely related to the presence of neutralizing antibodies to the virus in the serum. In man, examination of sera during the acute stage and during convalescence from influenza has revealed a constant rise of antibodies as a result of the infection (1–6). Further, evidence has been brought forward to suggest that individuals who escape infection after direct inoculation (7) or during an epidemic (3, 5) possess on the average a somewhat higher titer of antibodies than do those who acquire infection. In both the pig and the ferret it is readily demonstrable that the serum of a normal susceptible animal has no neutralizing antibody for influenza virus but that the serum of an animal convalescent from influenza virus infection has a high titer of neutralizing antibody (8, 9). However, in the case of the ferret it is difficult to accept the view that immunity to influenza virus is solely related to the possession of humoral antibodies. Immediately after infection the animal is solidly immune to a second inoculation of virus, but within from 3 to 6 months after infection reinoculation of virus again induces clinical evidence of infection although at this time antibodies, while perhaps less than before, can still be demonstrated in the serum (9, 10). Again, it is difficult, if not impossible, to induce complete immunity to intranasal inoculation of virus in the ferret by subcutaneous vaccination, although this procedure causes the development of antibodies (10, 11). Nevertheless, a second response to intranasal inoculation with influenza virus or the response of vaccinated ferrets is of a modified type and is usually not accompanied by the development of lung lesions. It has been suggested (6) that a high level of antibodies in the ferret assures immunity but that below this level susceptibility exists, and Hoyle and Fairbrother (3) are of the opinion that a similar state of affairs may hold in man.

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There are, however, certain difficulties in the general acceptance of this view of the mechanism of immunity to influenza. Soon after an infection by human influenza virus ferrets and mice are immune not only to the original virus but to other serologically different strains of influenza virus either of human or porcine origin, and at this time heterologous antibodies may be either low or unrecognizable in the serum (6, 10, 12). Moreover, pigs inoculated with swine influenza virus are subsequently immune to an inoculation with human virus although the serum after the first infection contains no antibody for the human virus (13, 14). On the other hand, while the majority of human patients have a very low level of antibody during the acute stage of influenza, some cases were encountered during the 1936–1937 epidemic with as high a level of antibody on the first day of the illness as was present in others during convalescence (4–6). Furthermore, certain individuals were observed (5) who, possessing little or no demonstrable antibodies against influenza virus, escaped the disease although subject to the same degree of exposure as others who incurred infection while retaining a comparatively high titer of circulating antibodies.

The foregoing data render it apparent that factors other than the mere presence of circulating antibodies as measured to date are required in order to explain the mechanism of immunity to influenza. The possibility immediately presented itself that some functional or anatomical factor in the respiratory tract itself played a rôle in the immunity to infection with influenza virus. With these facts in mind a study of the nasal mucosa of the ferret during infection and recovery from infection with influenza virus was begun.

Method of Examining the Nasal Structure in the Ferret

In order to compare the appearances in different ferrets it was necessary to use a standard method of histological examination. The ferret was sacrificed with carbon monoxide, the thorax opened, and the trachea ligatured. The great vessels were then cut, the head was severed from the neck, and the lower jaw cut off. The bony lateral walls of the nasal fossa were chipped away by bone forceps and the turbinates thus exposed on either side. A hacksaw was used to cut through the skull just posterior to the turbinates and the whole mass was then immersed in Zenker's solution to which glacial acetic acid had been added to a strength of 5 per cent. After 24 hours the mass was removed from fixative and by further chipping the turbinate system on each side of the nose was isolated. Fixing was then continued for another 24 hours. Dehydration and the embedding in paraffin were carried out in the usual manner. The turbinates from one side were cut horizontally and longitudinally; the other half was cut in two, the posterior portion discarded, and a transverse coronal section made. Sections were stained with hematoxylin and eosin, Giemsa, and Mallory's stain.

Normal Anatomy and Histology of the Ferret's Nose

A thorough study of the structure and variation in the nasal chambers of the normal ferret was an essential preliminary to the study of the variations and changes observed under the abnormal conditions of infection and repair.

The ferret's nose is filled with an intricate system of cartilaginous scrolls attached partly to the lateral wall of the nasal fossa and partly to the vertical wall separating the nasal fossa from the cranial cavity posteriorly. Anteriorly the turbinate scrolls are attached to a fibromuscular strand which continues forward in the form of a spiral uniting with the nostril and reducing the size of the nares to a narrow pore. Posteriorly the fossa communicates with the pharynx through a small oval opening. The nasal septum is cartilaginous anteriorly and bony posteriorly and is separated from the turbinates on either side by a mere chink. The accessory sinuses comprise the maxillary antra which form simple pockets on the lateral aspects of the turbinates and the floor of each antrum is occupied by a serous gland, the lateral nasal gland of the mammalia (15). In addition, numerous shallow outgrowths filled with cartilaginous scrolls are directed into the bone covering the posterior part of the roof of the fossa; these may represent frontal or ethmoid sinuses.

Fig. 1 shows above, the medial, and below, the lateral aspect of the turbinate system as exposed by the dissection employed. On the lateral aspect are visible the maxillary sinus, the lateral nasal gland, and the outlines of the turbinates still covered by the mucosa of the lateral wall of the fossa. The medial aspect which in the living animal faces the septum shows the division of the system into anterior and posterior portions by a well defined cleft. This can also be made out by reference to Fig. 2 which is an enlargement of a horizontal longitudinal section of one-half of the turbinates.

The anterior turbinates form a series of scrolls of cartilage covered by mucosa and attached both anteriorly and laterally to the walls of the fossa. Anterior to the lateral attachment the lateral wall of the fossa carries two ducts, the naso-lacrimal and nasal gland ducts which run forward to empty into the fossa between the anterior turbinates and the lateral wall. The posterior turbinate has a simpler structure and consists of parallel scrolls running horizontally from an attachment to the posterior wall of the fossa which is pierced by the olfactory nerve fibers. The anterior part of this turbinate undergoes an abrupt change of direction, gives off lateral branches, and may here be termed the middle turbinate (Fig. 2).

Histologically, respiratory and olfactory mucosae can be distinguished easily. The whole of the anterior turbinate, the middle turbinate, the antrum, the anterior half of the septum, and the lateral wall of the nasal fossa are lined by respiratory mucosa. Nearly all of the posterior turbinate is olfactory in structure except for occasional leaves which carry respiratory epithelium. Figs. 3 and 4 show the

detailed structure of the mucosa in the respiratory region through sections of the anterior turbinate and the mucosa covering the gland.   The respiratory epithelium is composed for the most part of a superficial layer of columnar ciliated cells and a deeper layer of flattened cells with darkly staining nuclei forming a pavement layer closely applied to the basement membrane.   Between these two layers intermediate cells may be interposed so that the epithelium is converted into a stratified columnar ciliated structure.   Such an epithelium is found over the septum and the lateral wall of the nasal fossa and in patches elsewhere, but most of the turbinate epithelium is composed of two layers.   In between the ciliated cells there are goblet cells which vary greatly in number in different ferrets.   Furthermore, in the normal ferret occasional intra-epithelial leucocytes or remains of leucocytic nuclei are found, especially near the tip of the nose.   The strand-like attachment of the anterior turbinate to the nostril is covered by a frankly stratified squamous though non-keratinized epithelium (Fig. 5), but such an epithelium does not occur in the normal ferret posterior to the junction of the attachment and the turbinate scrolls.   The only other variation from the columnar epithelium in the respiratory area is due to a shortening of the long diameter of the ciliated cell which occurs at the bends of the scrolls and produces the appearance of a cubical yet ciliated epithelium.

The submucosa or tunica propria of the respiratory area is very thin and consists only of connective tissue and venous sinuses except over the septum and lateral wall.   Here submucous glands occur but these are entirely absent from the turbinates proper.   Beneath the submucosa in both anterior and posterior turbinates is a thin lamina of cartilage.

The olfactory epithelium has a superficial columnar ciliated layer and beneath this are many cells with round nuclei, the olfactory cells (Fig. 6).   The submucosa of the olfactory area includes the glands of Bowman and also nerve fibers.

The lateral nasal gland is a compound tubulo-alveolar gland with serous secreting cells normally packed with secretion granules.   Accumulations of lymphocytes are sometimes found in the interstitial tissue of the gland and beneath the glandular epithelium.   The ducts of this gland and also of the lacrimal gland are lined by a simple columnar epithelium except near their anterior ends where stratified columnar epithelium is present.   The air passages between the turbinate scrolls constitute the turbinal passages and these are usually free from secretion but may occasionally contain a little mucus or cell debris with leucocytes.

The Nasal Lesion of Influenza Virus Infection in the Ferret

The material available for histological study was provided by a series of 26 ferrets 8 to 9 months of age, anesthetized with ether and inoculated intranasally on the same day with 2 cc. amounts of the same preparation of the PR8 strain of epidemic influenza virus in the form of a 1 per cent emulsion of infected ferret lung.   These animals were then sacrificed in pairs at various intervals up to 4

weeks after infection.   There were, in addition, a few ferrets inoculated for routine passage or other purpose with 5 or 10 per cent preparations of the PR8 strain of epidemic influenza virus, and 1 ferret similarly infected with the WS strain of virus.

In general the 1st day of fever corresponded with the 1st day after inoculation, the 2nd day of fever with the 2nd day after inoculation, and so on.   Figs. 8 to 26 represent the microscopical appearances in this group of ferrets.   They are all taken from coronal sections of the anterior turbinates.

Day 1.—2 ferrets were sacrificed 24 hours after inoculation before either had developed fever.   The appearance was normal in the gross, but microscopically a slight increase in the amount of fluid in the turbinate passages was seen (Fig. 8; compare with Fig. 7 taken from a normal ferret and viewed at the same power of magnification).   The epithelium also presented a slight increase in granularity and the cell outlines, including the cilia, were slightly blurred.   The goblet cells were seen to be emptying their contents into the fluid which formed a thin film over the epithelium.

Day 2.—The 2 ferrets sacrificed on this day had been febrile for 18 hours and macroscopically had injected, glistening turbinates.   Microscopically the turbinates showed necrosis of the respiratory epithelium, congestion, edema, and cellular infiltration of the submucosa, and the formation of an exudate in the turbinal passages (Fig. 9).   Almost the whole of the nasal respiratory area showed a complete desquamation of the ciliated columnar cells leaving only the basal "pavement" layer and basement membrane (Fig. 10).   Those parts of the respiratory mucosa, such as the lateral wall and the nasal septum, which were covered by stratified columnar epithelium showed in some areas a less complete desquamation, and one or two layers of polyhedral or cubical cells remained on the surface of the basal layer.   Elsewhere the basal layer formed the sole covering for the submucosa, although in each of the 2 ferrets a tiny pocket of unaffected ciliated columnar epithelium was found; in one instance this was in the middle turbinate region and in the other it was covering part of the gland in the region of the antrum.   The olfactory epithelium, the stratified squamous epithelium of the attachment of the anterior turbinates, and the columnar epithelium of the nasolacrimal and lateral nasal gland ducts were intact.   Those laminae in the posterior turbinates which were covered partly by respiratory and partly by olfactory epithelium showed an abrupt transition between the necrotic zone of the former and the normal structure of the latter.   The submucosa of the respiratory area showed a moderate congestion of the venous sinuses, edema of the interstitial tissue, and a mild infiltration of the latter with polymorphonuclear leucocytes and mononuclear cells.   The exudate in the nasal passages consisted of leucocytes, desquamated and necrotic epithelial cells, mucus, and debris.   The lateral nasal gland showed a discharge of secretion granules from the cells of the alveoli but no inflammatory changes.

Day 4.—The 2 ferrets were still febrile when killed on this day, and the gross appearance of their turbinates was the same as that of the previous ferrets.   Mi-

croscopically also the turbinates showed little change except that no ciliated columnar epithelium was present anywhere in the respiratory area. There was still exudate in the nasal passages although this was less dense than before. The respiratory area was still covered only by the basement membrane and basal cells. Here and there, however, commencing regeneration was seen, particularly over the lateral nasal gland, on the lateral wall of the nasal fossa, and along the epithelial stalk of the anterior turbinates (Fig. 11). In these areas flattened cells were beginning to accumulate so as to form a layer two or three cells thick. Mitotic figures were frequent in this layer and two cells in a state of anaphase are seen in Fig. 12, which is a high power view of the thickening epithelium. Elsewhere on the anterior turbinates the absence of a reparative process was conspicuous. The submucosa showed a dense cellular infiltration with polymorphonuclear leucocytes, mononuclear cells—lymphocytes, plasma cells, and monocytes—and fibroblasts. The venous sinuses were still engorged. The lateral nasal gland showed a slight increase in lymphocytes in the interstitial tissue and in the submucosa, and the alveolar cells were in a state of active secretion.

Day 6.—The ferrets sacrificed on the 6th day were already convalescent with normal temperature, and the nasal turbinates were almost normal macroscopically. Microscopically, repair of the epithelium was in full progress, although there was still considerable exudate in the nasal passages and the submucosa was still densely infiltrated. The turbinates of one ferret (Fig. 13) appeared to be slightly less advanced in repair than that of the other (Figs. 14 and 15). Both showed, however, substantially similar changes. The epithelium throughout the respiratory area was two, three, or four cells thick, and formed a structure similar to the transitional epithelium of the urinary tract. The deep cells of the layer were cubical or polyhedral in shape, but the superficial cells were elongated and flattened, and their cytoplasm seemed to form a continuous layer bounding the free surface of the epithelium. The nuclei of this epithelium were hyperchromatic when compared with those of normal columnar cells. Occasional leucocytes and fragments of the nuclei of leucocytes were seen in between the epithelial cells.

In a few pockets of the anterior turbinates of the ferret showing the more advanced repair a remarkable type of epithelium was present. This seemed to be the result of increased flattening of the superficial cells. As a result, the epithelium presented an appearance suggesting stratified squamous epithelium. The deep cells were arranged in a palisade fashion, the intermediate cells were polygonal in shape, and the superficial cells were flattened but non-keratinized squamae. It was not possible to decide definitely whether the repair of the turbinate epithelium had been accomplished by cell migration from the squamous epithelium at the tip of the nose or by multiplication of the basal layer, but the uniform character of the repair and the absence of any advancing edge of epithelium were considered to favor the latter process.

Beneath the epithelium two changes were evident. Fibroblasts were replacing

the mononuclear and polymorphonuclear cells in the interstitial tissue and were accumulating on either side of the cartilage as though to enclose it in a fibrous capsule. The cartilage itself was also undergoing change. Here and there throughout the course of the laminae there were multinucleated chondroclasts which appeared to be attacking the cartilage and breaking it up into strips.

Days 7 and 8.—The turbinates of the 2 ferrets sacrificed when convalescent on the 7th and 8th days respectively were covered by a stratified squamous epithelium of ovoid cells lying parallel to the surface, only a few laminae being still covered by an epithelium with polygonal superficial cells. Figs. 16 and 17 from the 7th day ferret show the striking appearance of the squamous type of epithelium. The superficial layers were undergoing desquamation partly by flaking off and partly by the formation of epithelial blisters such as those from the 8th day ferret seen in Figs. 18 and 19.

This latter ferret showed in addition, however, occasional laminae where the repair had reached a more advanced stage. Here the deepest layer of cells had flattened and the superficial cells were becoming differentiated so that their cytoplasm was ranged at right angles to the basement membrane, thus producing a columnar epithelium mounted on several layers of cells (stratified columnar). The nuclei of the superficial cells were less hyperchromatic than before. It was not possible to decide exactly how this stage was reached. Possibly the casting off of the superficial layers was simultaneously accompanied by a differentiation of the cells beneath into the columnar type of cell, or it may be that both processes were due to the increase in pressure occasioned by the accumulation of cells in the developing mucosa.

The three stages, however, of transitional, stratified squamous, and stratified columnar epithelium were clearly visible side by side at the 8th day although most of the epithelium was of the stratified squamous type. The repair of the submucosa had also reached a more advanced stage by this day. Fibroblasts were becoming converted into chondroblasts and these cells bordering on the cartilage can be clearly seen in Fig. 18. Elsewhere fibroblasts were scattered throughout the interstices of the submucosa and beneath the basement membrane of the epithelium. There was still a loose exudate composed of mucus and leucocytic debris in the nasal passages. Leucocytes were still visible between the epithelial cells but not to any extent in the submucosa. There was still an infiltration of mononuclear cells in the gland, particularly in the submucosa.

Day 10.—By the 10th day (Fig. 20) most of the respiratory epithelium showed a columnar layer of superficial cells which in places were developing cilia. However, there were still areas of stratified epithelium in the anterior turbinates and in other areas several abnormalities were noted: (*a*) Near the tip of the nose the epithelium had an irregular outline with fronds of hyperplastic cells projecting outwards; (*b*) in many areas there were oval or round cavities in the epithelium often containing a few leucocytes (Fig. 21); (*c*) beneath the newly formed columnar

cells there were one, two, or three layers of polygonal or cubical cells with hyperchromatic nuclei so that the epithelium was a stratified columnar one; and (d) goblet cells were still undeveloped. The submucosa was more normal and the fibroblasts lining the cartilage were being converted into chondroblasts by a process of encapsulation. There was still a scanty leucocytic exudate in the nasal passages, but there were fewer intra-epithelial leucocytes than formerly.

Days 13 and 14.—Macroscopically the turbinates at this stage appeared velvety, and microscopically the epithelium was fully ciliated, but hyperplastic and stratified columnar in type. Goblet cells were beginning to reappear. However, near the tip of the anterior turbinate there were still epithelial fronds, and intra-epithelial cavities were still prominent in many parts of the mucosa although not in the area shown in Fig. 22. Many of the intra-epithelial cavities still contained leucocytes; others, however, showed a reorientation of their surrounding cells as though to form an alveolus, and cilia were growing on the inner rim of the alveolus. There were other areas which resembled superficially intra-epithelial cavities composed of three or four goblet cells massed together, a formation occasionally seen in the normal ferret's mucosa. The intra-epithelial alveoli, however, have never been seen in normal ferrets. There was still an exudate in the nasal passages but this was much scantier than before. Many fibroblasts still persisted (Fig. 22).

Days 16 and 17.—Fig. 23 shows that the epithelium was much more normal in appearance by the 17th day. This was due to disappearance of the intermediate cells between the columnar and basal cells and to a flattening out of the basal layer. Fronding of the epithelium was still present at the tip of the nose, however, and at the extreme tip of the laminae of the anterior turbinate there was a non-ciliated pseudostratified epithelium two cells thick in which both superficial and basal cells were cubical in shape. Exudate had at this time disappeared. Cartilage regeneration was still active and with its formation the thickness of the submucosa had diminished. The nasal gland was secreting less actively and the mononuclear infiltration of its submucosa was replaced by fibroblasts.

Days 21 and 28.—The noses of the ferrets sacrificed 21 days after infection might almost be considered normal. The epithelium was ciliated columnar, the submucosa was thin, the gland was perfectly normal, and the cartilage was well formed. More careful search, however, revealed certain abnormal appearances which were still visible both in these ferrets and in those studied 28 days after infection. The epithelium at the extreme tip of the anterior turbinates retained a pseudostratified non-ciliated appearance with two or three layers of polygonal or cubical cells. Passing from the attachment of the anterior turbinates backwards, one encountered first a continuous layer of stratified squamous epithelium, next a pseudostratified layer two cells deep, and then a ciliated columnar layer. Nevertheless, occasional patches of pseudostratified epithelium could be made out some distance away from the tip (Fig. 24, taken from a 28 day ferret). Epithelial irregularities persisted with stretches of stratified ciliated columnar cells, epithelial fronds, and intra-epithelial cavities lined by cilia. The submucosa was still

slightly thicker and more fibrous than normal. Cartilage formation was still active and a peculiar staining effect was noted with hematoxylin, the exact significance of which was doubtful. Parts of the cartilage, often near the center of the laminae, but sometimes at the side (Fig. 25), were a deeper blue in color than the rest. There was a sharp line at the junction of the two types of cartilage and it seems likely that this effect was due to the reformation of cartilage which occurred during the repair process. The line may be analogous to the growth lines which occur in the bones of children as a result of arrested formation of bone during an acute infection. It has not been possible to determine how long such lines in the cartilage persist, but they were not seen in ferrets killed 4 months or 6 months after an attack of influenza, although they were visible in a few ferrets killed 3 months after an attack. While the possibility that they represent restoration of normal growth cannot be excluded, the lines have not been observed in normal ferrets but are practically pathognomonic of the process of repair.

The above findings in the series of 26 ferrets infected with the virus of epidemic influenza provided a picture of the orderly development and repair of the lesion produced in the nose by this strain of virus. The remaining few ferrets which were studied provided additional data relating to the acute lesion and its repair. 3 ferrets aged 18 to 20 months, which were a year older than the ones previously studied, were sacrificed on the 3rd day of an infection with the PR8 strain of virus. The histological appearances did not differ significantly from those seen in the younger ferrets, so that within the range studied the histology of the acute lesion of influenza did not seem to vary with the age of the ferret.

One ferret in the series had been inoculated with the WS strain of human influenza virus and was sacrificed on the 3rd day of the infection. The respiratory mucosa of this animal showed the same changes as those seen in ferrets infected with the PR8 strain of virus. However, there was slightly more exudate to be seen and an important difference was the fact that a necrosis of the olfactory epithelium occurred on a few of the laminae (Fig. 26). As the PR8 strain of virus caused no demonstrable damage to the olfactory mucosa, while the one ferret infected with WS strain showed partial necrosis of this tissue, it is suggested that different strains of epidemic influenza virus may vary somewhat in their specificity and in the extent of the damage they produce.

DISCUSSION

The foregoing observations have revealed an orderly progression of changes in the nasal mucous membrane of ferrets after intranasal inoculation of relatively large doses of epidemic influenza virus. After 24 hours little but irritative phenomena is observed while after 48 hours complete destruction of the respiratory epithelium has occurred.   At that time the respiratory area is covered only by the thin basement membrane; the submucosa is edematous and infiltrated with inflammatory cells; there is a rich exudate chiefly polymorphonuclear in type in the air passages.   Illustrating the sharp specificity of the lesion the olfactory mucous membrane, on the other hand, exhibits practically no evidence of injury.   In the next 2 days no further damage occurs and one gains the impression that a phase between injury and repair has been reached.

By the 6th day reparative processes have definitely begun and over the respiratory area a transitional type of epithelium composed of polyhedral cells three to four layers deep is comparatively uniform. In the next 48 hours a rapid advance results in the development of a further differentiated epithelium predominantly stratified squamous in type, with desquamation of superficial squamae and the formation of epithelial blisters.   In some places the deeper cells are flattening and the superficial cells are suggesting the return of a columnar epithelium.   Moreover, hyperplastic papillary folds of the regenerating epithelium are being thrown up.   2 days later the epithelium approaches a stratified columnar type and cilia have begun to appear. The papillary fronds are much more marked and intra-epithelial nests of columnar cells are caught in the hyperplastic epithelium.   At this time the exudate is decreased in amount, the inflammation of the submucosa is subsiding, and a marked acceleration of cartilage formation has begun.

By the 13th or 14th day ciliated epithelium occupies most of the affected area.   Hyperplasia is still prominent, however, and islands of transitional epithelium remain.   Goblet cells are seen for the first time.   Only a scanty exudate is present.   On the 16th to 17th days the sections reveal a still closer approach to normal.   The epithelium is columnar in type and the basal cells have become

flattened. Goblet cells are extremely numerous and active. Exudate is no longer present. It is interesting, however, that the anterior tip of the respiratory area is still non-ciliated. In the 3rd and 4th weeks the respiratory epithelium is essentially normal except for pseudostratified non-ciliated patches of cells which remain here and there directly adjacent to normal ciliated columnar epithelium. Moreover, hyperplastic fronds and intra-epithelial cavities are still observed. The submucosa is somewhat thicker and more fibrous than normal while the laminal cartilage shows dark staining zones of accelerated cartilage formation. After 4 to 6 months nothing more than suggestive residues is noted.

The damage produced by epidemic influenza virus in the nose of the ferret is striking in its specificity for the respiratory epithelium and in the totality with which it destroys that structure. Only rarely in the previously untreated animal is any respiratory epithelium left, while the olfactory epithelium is essentially untouched. Despite the severity and the completeness of the damage, repair is well under way by the 6th day after infection, and between the 6th and 14th days the regenerating epithelium progresses from a relatively undifferentiated transitional epithelium of polygonal cells, through a well organized stratified squamous type resembling the normal lining of the nasal vestibule, to a hyperplastic stratified columnar epithelium which rapidly develops cilia. The character of the regenerating respiratory membrane is so manifestly different from that of the normal tissue as to suggest immediately the possibility of different functional reactions such as described by MacNider (16–22) in the liver and kidneys of dogs previously subjected to chemical injury. Moreover, the cycle of epithelial changes observed in the present experiments is somewhat paralleled by the observations of Boling (23) upon repair after traumatic damage to the nasal mucous membranes of sheep. In the experiments of MacNider and Boling the alteration in cell types resulted in resistance to subsequent injury by chemical agents and it seems not unlikely that a similar resistance might be exhibited by the regenerating respiratory membrane of ferrets following influenza virus infection. The second paper of the series deals with experiments designed to test this hypothesis.

SUMMARY

A study has been made of the nasal histology in normal ferrets and in ferrets during and after infection with epidemic influenza virus. During the acute stage of infection the respiratory epithelium of the nasal mucous membrane undergoes necrosis with desquamation of the superficial cells and exudation into the air passages, and an inflammatory reaction occurs in the submucosa. Repair begins on the 4th day after infection, and from the 6th to the 14th day the respiratory area is covered successively by a transitional, a stratified squamous, and finally a stratified columnar epithelium. By the 21st day after infection the epithelium has been largely restored to normal but repair in the submucosa and cartilage is still in progress. The respiratory mucosa is substantially normal in structure 1 month after infection although minor abnormalities of cellular arrangement and type can still be distinguished.

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EXPLANATION OF PLATES

All sections stained with hematoxylin and eosin.

PLATE 36

FIG. 1. The turbinate system in a normal ferret exposed from the medial aspect (above) and from the lateral aspect (below). Approximately × 2. The tip of the nose lies to the right and the roof of the nasal fossa above each half. The medial aspect shows the anterior turbinate to the right and the posterior turbinate to the left separated by a cleft. The lateral aspect shows the antrum and lateral nasal gland on the surface of the turbinates. A bristle has been inserted through the torn roof of the antrum.

FIG. 2. Horizontal section of left turbinate system in a normal ferret with the tip of the nose pointing upwards. Approximately × 3.

1. Anterior turbinate ⎫ respiratory epithelium.
2. Middle turbinate   ⎬
                      ⎭
3. Posterior turbinate, olfactory epithelium.
4. Lateral nasal gland.
5. Cavity of antrum.

FIG. 3. Normal respiratory epithelium in the anterior turbinate. × 170. The lamina of cartilage is covered by a ciliated columnar epithelium resting on a thin submucosa containing venous sinuses.

FIG. 4. Normal respiratory epithelium covering the lateral nasal gland. × 570. Ciliated columnar and goblet cells comprise the superficial layer of the epithelium. The gland is composed of serous acini.

FIG. 5. Normal epithelium covering the anterior attachment of the anterior turbinate. × 570. A stratified squamous epithelium without keratinization.

FIG. 6. Normal olfactory epithelium covering the posterior turbinate. × 80. A multilayered epithelium with superficial ciliated cells and deeper olfactory and supporting cells. Nerve fibers run in the submucosa.
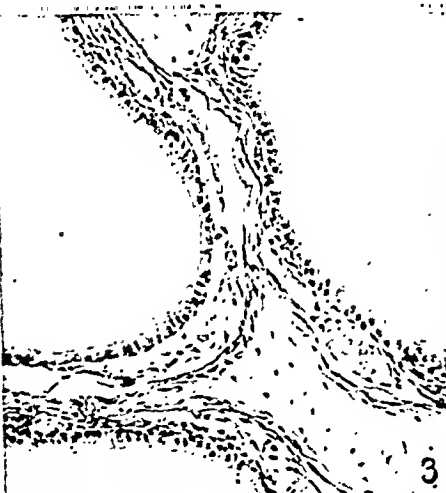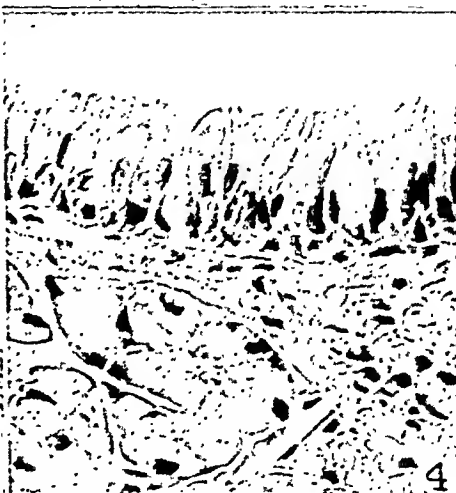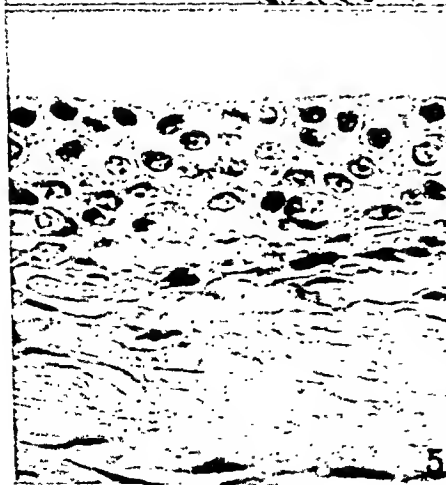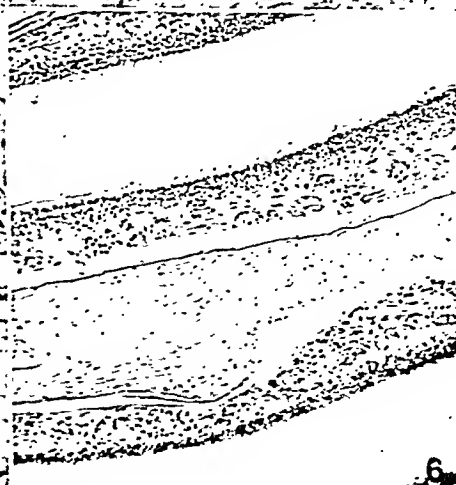
(Francis and Stuart-Harris: Nasal histology of influenza infection. 1)

PLATE 37

FIG. 7. Normal anterior turbinate. × 80. Regular ciliated columnar epithelium. Air passages free from exudate.

FIG. 8. Influenza. Day 1, 24 hours after infection. Anterior turbinate. × 80. Ciliated columnar epithelium covered by a thin film of exudate in the air passages (mucus and leucocytes). Inclusion bodies were not seen in specially stained preparation.

FIG. 9. Influenza. Day 2. Anterior turbinate. × 80. The epithelium has been desquamated. The air passages are filled with exudate. The submucosa is wider than normal. Inclusion bodies were not seen in specially stained preparation.

FIG. 10. High power view of Fig. 9. × 570. The epithelium is a single layer of flattened cells incorporated with the basement membrane. The submucosa is edematous and infiltrated with polymorphonuclear leucocytes and mononuclear cells. The exudate in the air passages is composed of leucocytes and cell debris.

FIG. 11. Influenza. Day 4. Anterior turbinate. × 80. The epithelium is still a single flattened layer except along the stalk of the anterior turbinate shown to the right where accumulation of cells is in progress. The submucosa shows a richer cell infiltration and is still acutely inflamed Inclusion bodies were not demonstrated.

FIG. 12. High power view of Fig. 11. × 570. Shows the earliest sign of regeneration of epithelium on one side of a lamina (below). The arrows point to two nuclei showing mitosis (anaphase). The epithelium on the other side of the lamina is probably a single layer of cells but has been cut obliquely.

(Francis and Stuart-Harris: Nasal histology of influenza infection. I)

PLATE 38

Fig. 13. Influenza. Day 6. Anterior turbinate. × 80. Epithelial rege eration is in progress, the submucosa is densely cellular, and fibroblasts are formir a layer alongside the cartilage which is being split by chondroclasts. Exudate sti in the air passages.

Fig. 14. Influenza. Day 6. Anterior turbinate. × 80. Slightly mor advanced stage of epithelial regeneration.

Fig. 15. High power view of Fig. 14. × 570. The epithelium is composed of polyhedral and cubical cells with hyperchromatic nuclei. It is a stratified transitional type of structure.

Fig. 16. Influenza. Day 7. Anterior turbinate. × 80. The epithelium is now a stratified squamous structure, the superficial layers of which are being desquamated.

Fig. 17. High power view of Fig. 16. × 570. Note the desquamation of the superficial squamae and hyperchromatic nuclei.

Fig. 18. Influenza. Day 8. Anterior turbinate. × 80. Most of the epithelium in this turbinate was stratified squamous as in Fig. 16 but this area was chosen to illustrate the formation of epithelial blisters. Cartilage regenera- tion is beginning in the submucosa.

Fig. 19. High power view of Fig. 18. × 570. The blisters are composed of intra-epithelial cavities containing leucocytes. Note the palisade type of forma- tion in which the deep cells of the epithelium are arranged.
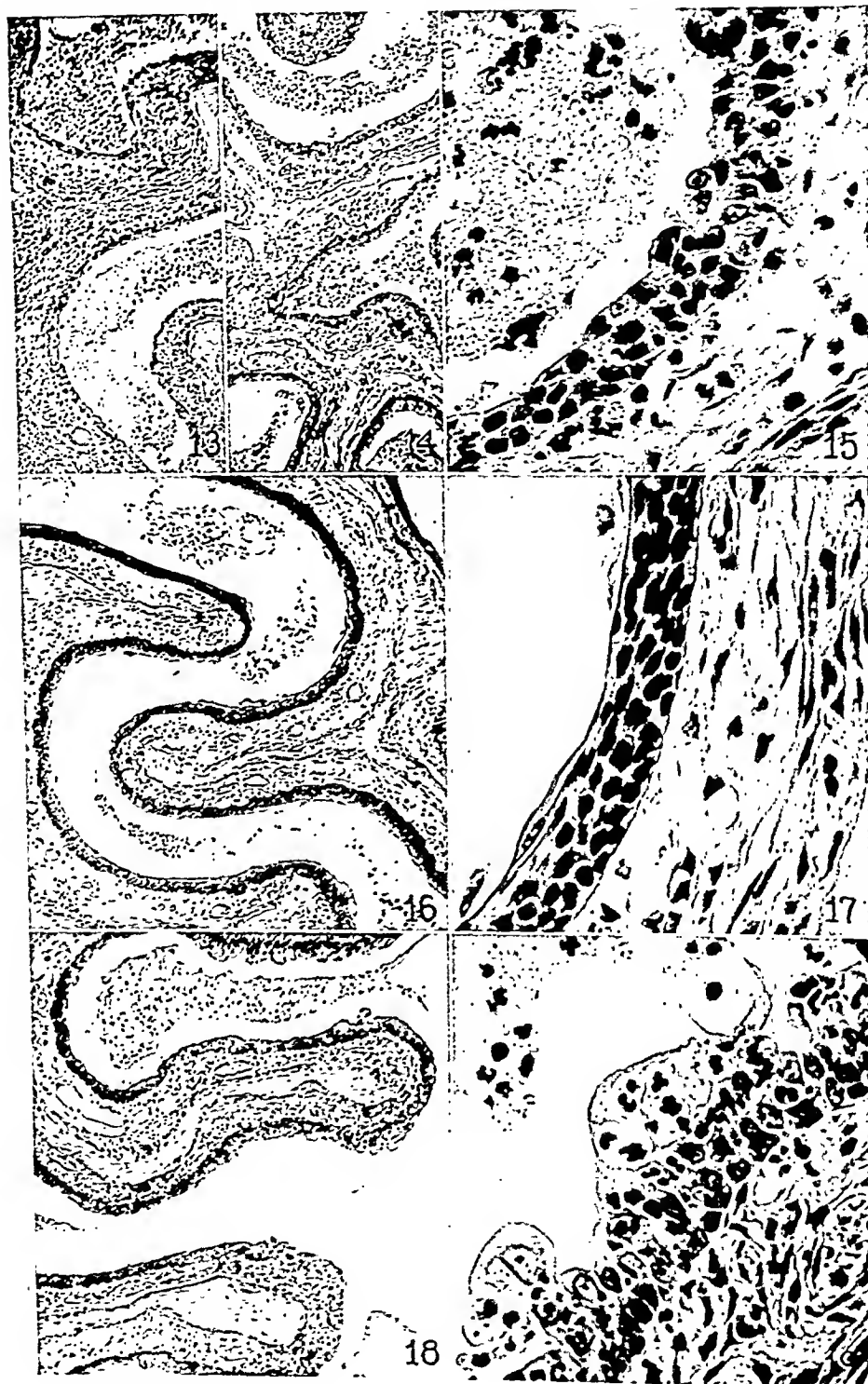
Photographed by Joseph B. Haulenbeck

(Francis and Stuart-Harris: Nasal histology of influenza infection. I)

PLATE 39

FIG. 20. Influenza. Day 10. Anterior turbinate. × 80. The epithelium is beginning to appear more normal. The first columnar elements are visible but there are still several layers of cells so that the epithelium is stratified columnar. There is still a small amount of exudate in the air passages.

FIG. 21. High power view of Fig. 20. × 570. Shows the earliest formation of columnar cells with young cilia sprouting from the free surface. Below, an intra-epithelial cavity is shown which contains leucocytes. The submucosa shows a layer of fibroblasts next to the cartilage and some of these are in the process of transformation into chondroblasts.

FIG. 22. Influenza. Day 14. Anterior turbinate. × 80. The epithelium is hyperplastic and stratified columnar ciliated in type. The laminae of cartilage are widening as new cartilage is laid down from fibroblastic transformation. The air passages are free from exudate.

FIG. 23. Influenza. Day 17. Anterior turbinate. × 80. The epithelium is simple columnar ciliated in type and contains abundant goblet cells.

FIG. 24. Influenza. Day 28. Anterior turbinate. × 570. A portion of pseudostratified non-ciliated epithelium is shown which was present near the tip of the anterior turbinate. The epithelium is mostly of a normal type, however.

FIG. 25. Influenza. Day 28. Anterior turbinate. × 80. Cartilage regeneration is a striking feature of this section. The deeply stained zone is apparently older than the lighter pink-staining area to the left of the section. Note the shrinking of the submucosa which has occurred as the cartilage has been laid down.

FIG. 26. Influenza. WS strain of virus. Day 3. Posterior turbinate. × 570. The PR8 strain of virus does not affect the olfactory epithelium but some of the laminae in the posterior turbinate of this ferret infected with the WS strain showed epithelial necrosis. The absence of a basement membrane in this area has led to the exposure of the nerve endings and glands of the submucosa.
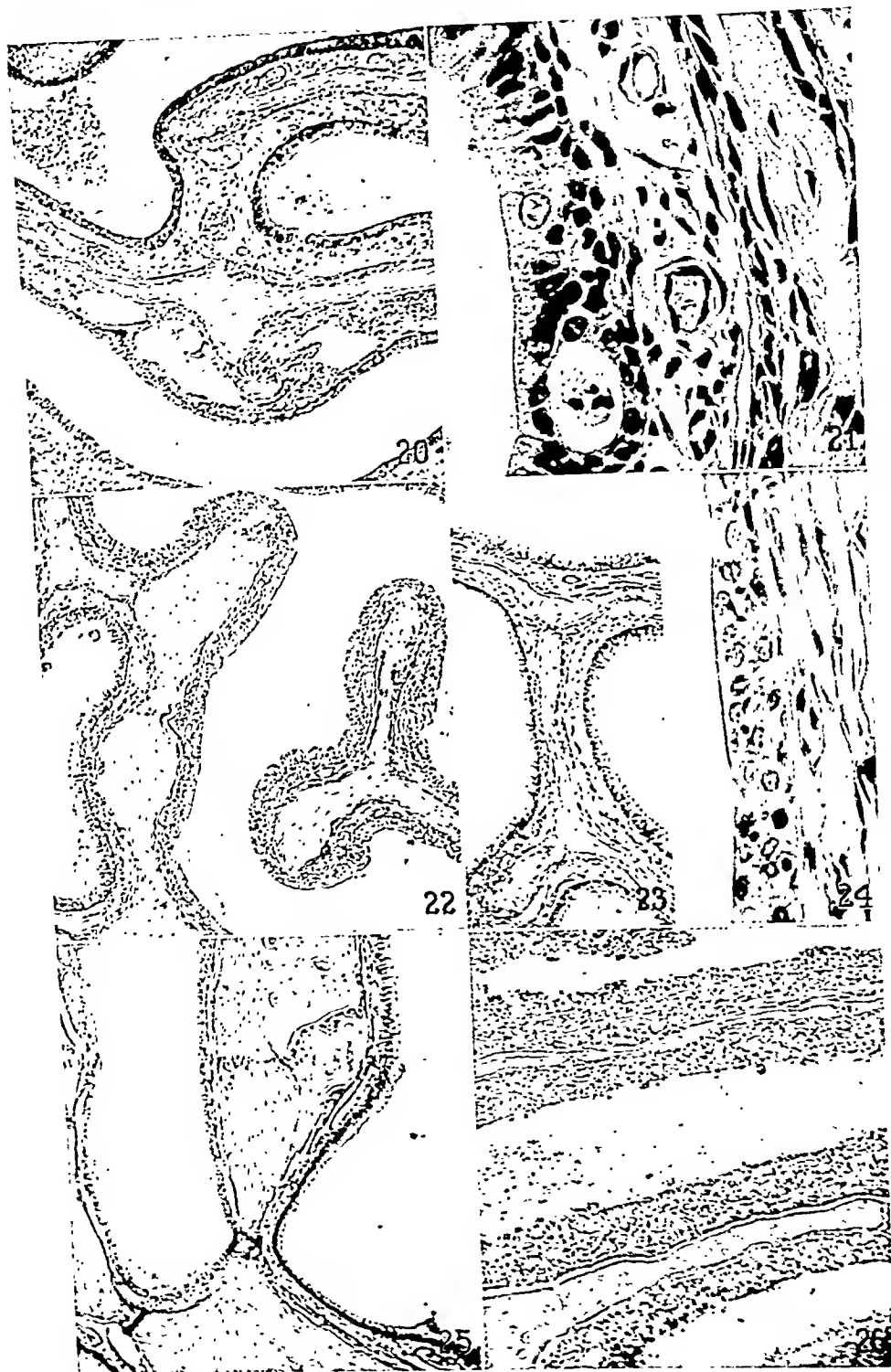
(Francis and Stuart-Harris: Nasal histology of influenza infection. I)

STUDIES ON THE NASAL HISTOLOGY OF EPIDEMIC INFLUENZA VIRUS INFECTION IN THE FERRET

II. The Resistance of Regenerating Respiratory Epithelium to Reinfection and to Physicochemical Injury

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PLATES 40 AND 41

Iontophoresis of the nose, using a weak galvanic current and a solution of 1 per cent zinc sulfate, has been shown to cause a disintegration of the nasal mucosa, an inflammatory reaction in the submucosa, and hyperemia of the venous sinuses (1, 2). Boling (3) also showed that if zinc iontophoresis was applied in sheep to a portion of the nasal mucosa which included an area of epithelium regenerating after traumatic removal, this area alone was uninjured. He concluded that the stratified cells covering a repairing mucosa were resistant to injury and were even stimulated to more rapid development as evidenced by an increased number of mitoses. Because of the similarity of the repair process following influenza virus infection (4) and that described by Boling, it was of interest to study the resistance in the former instance to specific and non-specific injury. Using a technique similar to that employed by Boling, the effect of intranasal zinc iontophoresis was studied in normal ferrets and in ferrets convalescent from an attack of influenza.

Experimental Methods

The procedure used was to anesthetize the ferret with one or more intraperitoneal injections of evipal[1] (5.0 cc. of a 1 per cent solution was usually sufficient),

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[1] Sodium salt of *n*-methylcyclohexenylmethyl barbituric acid.

and then to instil a few drops of 2 per cent cocaine into each nostril. The anode, a fine platinum-iridium wire 1 mm. in diameter, was inserted into one nostril and a gentle stream of 1 per cent zinc sulfate solution was introduced by gravity from a reservoir into the other nostril through a fine intravenous needle inserted into the nostril. The cathode, a sheet of copper covered with gauze and moistened with saline, was fastened over a clipped area of skin on the back. After several trials with different strengths of current the following procedure was adopted: A current of 3 milliamperes at between 8 and 10 volts was passed for 2½ minutes; the anode and irrigating needle were then reversed and the current was again passed for a further 2½ minutes. In this way both sides of the nose received identical treatment. Aspiration of the irrigating solution into the lung was minimized by inserting a bent glass tube over the back of the tongue so as to drain the pharynx. The ferrets usually regained consciousness ½ to 1 hour after the operation.

The dissection of the nose and the preparation of sections were carried out as in the preceding paper (4). The tests for resistance to reinfection with influenza virus were made by the intranasal inoculation of a 10 per cent suspension of PR8 strain of virus.

The Effects of Ionization on the Normal Nasal Mucous Membrane

In previously untreated ferrets sacrificed 3 or 4 hours after intranasal ionization the turbinates appeared hemorrhagic and darkened. Microscopically almost the entire epithelium of the nose, both respiratory and olfactory, showed a coagulative necrosis. At this stage, the epithelium appeared to be lifted off the basement membrane and had the hazy outline of coagulated tissue. The basal cell layer, however, was intact. The submucosa showed intense hyperemia and diapedesis of polymorphonuclear leucocytes, and a purulent exudate was already present in the nasal passages.

In the following series of ferrets, the nose was usually examined on the 1st day after ionization because the coagulated epithelium had then been desquamated and removed so that the gross appearance was less obscured by the dead tissue. At this stage the changes in the respiratory area (Fig. 1) were very like those present in the nose on the 2nd day of influenza. The high power view (Fig. 2) shows the exudate, the desquamated epithelium with intact basal layer, and the inflammatory changes of the submucosa. In contrast, however, to the absence of damage to the olfactory epithelium as a result of infection with the PR8 strain of influenza virus, zinc ionization resulted in a complete desquamation of the entire epithelium in the olfactory area. The congestion and inflammatory changes were the same as those seen in the respiratory area and Bowman's glands were stimulated to increased secretion. Having thus established the picture produced by ionization in normal ferrets, the same technique was next employed in ferrets convalescent from influenza virus infection.

*The Effect of Ionization or Reinfection on the Respiratory
Mucous Membrane of Convalescent Ferrets*

A series of ferrets was subjected to intranasal ionization with zinc sulfate at various intervals during convalescence from experimental influenza, and sections were prepared from the turbinates 24 hours later (Table I). Since the primary purpose was to test the resistance of the regenerating respiratory epithelium, the earliest tests were carried out in ferrets 7 to 8 days after infection, at which time a well developed transitional or stratified squamous type of epithelium covers the respiratory area. At this stage of convalescence reinoculation of influenza virus into the nose elicited no detectable reaction either clinically or anatomically. In

TABLE I

*Results of Ionization on the Nasal Mucosa of Ferrets at Different Intervals after
Infection with Influenza Virus*

Ferret No.	Day after infection	Result
11-06*	7th	No effect
11-18	7th	" "
10-95	8th	" "
10-94	15th	Partial necrosis of respiratory mucosa; complete necrosis of olfactory mucosa
10-78	21st	Partial necrosis of respiratory epithelium; complete necrosis of olfactory epithelium
11-11	28th	Almost complete necrosis of respiratory epithelium; complete necrosis of olfactory epithelium
11-03	38th	Almost complete necrosis of respiratory epithelium; complete necrosis of olfactory epithelium

* Examined immediately.   All other ferrets examined 24 hours after ionization.

Figs. 3 and 4 is shown a section from the turbinate of a ferret (11-01) tested with virus intranasally on the 8th day and sacrificed on the 11th day after the original infection. The epithelium revealed no evidence of new damage but presented a state of repair characterized by stratified columnar epithelium with early cilia formation. The state of repair was quite typical of that seen on the 10th and 11th days after a single inoculation. A control ferret (10-99) in its 8th day after primary infection with virus was sacrificed for comparison at the same time the previous animal was given its second virus inoculation. It revealed a repair process (Figs. 5 and 6) typical of the 8th day of convalescence with epithelium in the phase of change from transitional to stratified columnar type.

Since it was shown that the abnormal transitional epithelium of the convalescent nasal respiratory mucous membrane was completely

resistant to reinoculation of the virus, it was of further interest to determine whether this resistance was specific for the virus. If, however, the resistance of the regenerating epithelium should be effective against destructive agents of an entirely different nature, such as that furnished by ionization with zinc sulfate, it would represent an instance of tissue immunity or refractoriness unrelated to resistance in the ordinary immunological sense. Consequently ferrets were tested by intranasal ionization at different periods in convalescence from influenza virus infection.

Ferrets treated by ionization on the 7th and 8th days after infection with influenza virus behaved just like those receiving a second inoculation of virus. There was no evidence of damage to the nasal mucosa as a result of the procedure. Sections of ferret 10-95 (Figs. 7 and 8) revealed a picture quite comparable to the non-ionized control (Figs. 5 and 6) except that repair in the latter seemed slightly less advanced due probably to a difference of 24 hours in the time of death. Moreover, in contrast to the widespread damage to the olfactory epithelium created by ionization in the normal ferret, the convalescent ferret on the 7th to 8th day showed complete absence of reaction in the olfactory area.

In order to exclude the possibility that ionization at this stage of repair of the influenzal nasal lesion might produce only a slight transient damage which would be repaired in the 24 hour period between ionization and the removal of the turbinates, the nasal passages of 2 ferrets (11-06 and 11-18) were subjected to ionization on the 7th day of influenzal infection. One was sacrificed immediately, the other after 24 hours. Sections of neither showed evidence of recent necrosis or damage, while the repair of the influenzal virus lesion was in both instances in approximately the same stage.

These results show clearly that the resistance of the abnormal epithelium present in the respiratory area of the nose on the 7th to 8th day after influenza virus infection is extremely effective and is non-specific. The refractory state of the epithelium withstands further damage by the original destructive agency and furthermore is completely resistant to a physicochemical agent which in the normal ferret creates a widespread destruction.

After this stage in the convalescence from influenza virus infection different effects were noted.

While the epithelium of the nose was unaffected by reinoculation of virus during the 3rd and 4th weeks of convalescence (see ferrets 10-43, 10-44, 10-46, 10-50, and 10-52 in the third paper of this series (5)), varied degrees of damage were occa-

sioned by the ionization process. Thus on the 15th day of convalescence, when the non-ionized control (10-98, Fig. 9) revealed a stratified columnar ciliated epithelium, ionization resulted in a partial desquamation of the epithelium (10-94, Fig. 10). While the residual epithelium was two, three, or four cells thick the ionization seemed to have effected the removal of most of the superficial columnar cells, leaving the intermediate and basal cells intact (Fig. 11). Nevertheless, comparatively large numbers of ciliated cells escaped.

Ionization on the 21st day after infection produced a picture similar in some places to that at 15 days. Desquamation of the superficial ciliated cells had occurred although one or two of the deeper cell layers remained intact. In other areas, however, the destruction was almost as extensive as in the normal ferret after ionization in that the epithelium had been shed leaving only the basement membrane and pavement epithelium. Similar effects were obtained in a ferret subjected to ionization 28 days after virus infection (Fig. 12) in which the response resembled that of the normal ferret. The epithelium had been shed, the submucosa was infiltrated with inflammatory cells, and an exudate had formed. There were, however, both in this ferret and in the one treated on the 38th day of convalescence surviving patches of epithelium of a ciliated columnar type which had not been affected by ionization. While it is true that in the previously untreated ferrets occasional patches of ciliated columnar epithelium remained intact after the ionization process was applied, they were never as extensive as in the convalescent influenza ferrets treated in the same manner.

These observations reveal clearly that on the 8th day of convalescence from influenza virus infection, the polygonal transitional or stratified squamous epithelium which covers the nasal respiratory area is resistant to reinfection and to physicochemical injury. In the next weeks, however, while still resistant to reinfection with virus, an increasing susceptibility of the respiratory mucous membrane to chemical injury is noted until the 4th to 5th weeks when the damage induced by ionization is nearly as severe as that produced in the normal ferret.

During the period between the 8th and 15th days in addition to the mere aging a distinct maturation of the respiratory epithelium takes place in which the rather primitive transitional stratified epithelium is giving way to the highly differentiated ciliated columnar cells. These facts plus the observation that the latter are the cells most definitely affected suggest that it is the ciliated columnar cell which is the one susceptible to ionization and that resistance is related to the primitive undifferentiated cell type.

The Effect of Ionization on the Olfactory Mucous Membrane of Convalescent Ferrets

A curious effect was produced on the olfactory epithelium of the convalescent ferret by ionization. It has already been stated that the PR8 strain of influenza virus produces no visible effect on this epithelium and therefore if resistance to ionization were a property of certain types of cells, it might be expected that the olfactory epithelium of the convalescent ferret would be as susceptible to the necrotizing effect of ionization as the olfactory epithelium of a normal ferret. In fact, the olfactory epithelium of the 7 and 8 day convalescent ferrets was unharmed by ionization, but that of the convalescent ferrets ionized on the 15th day or after was completely necrotized by the ionization. In the convalescent ferret so long as no damage due to ionization occurred in the respiratory area, the olfactory mucosa escaped; but at a time when the respiratory mucosa again became susceptible to damage, the olfactory mucosa responded in the same way as that of a normal animal. Two explanations may be suggested for this phenomenon. First, it may be that destruction of the superficial cells of the respiratory mucous membrane exposes the contiguous olfactory cells which are then destroyed, whereas when the respiratory epithelium is resistant, this phenomenon exerts a protective effect upon the olfactory epithelium. On the other hand, since the WS strain of influenza virus in one instance did cause damage to the olfactory epithelium, it may be that the PR8 strain attacks the cells without causing obvious injury but thereby inducing a refractory state protective against chemical injury.

The Effect of Zinc Sulfate Alone on the Nasal Mucous Membrane

Further confirmation of the results obtained by the technique of zinc ionization was found in a study of the effect of prolonged intranasal irrigation with zinc sulfate solution. The 1 per cent solution used during ionization was itself damaging to the nasal mucosa of the normal ferret, and irrigation alone under anesthesia for 15 minutes caused as complete a desquamation of the epithelium as ionization. When the nostrils of convalescent ferrets were irrigated for 15 minutes, the repairing epithelium was unattacked by the zinc sulfate on the 8th and 9th days, partially damaged on the 16th and 19th days, and more extensively damaged on the 28th and 29th days. In these latter ferrets whose noses were irrigated 1 month

after infection, foci of resistant ciliated epithelium were found in the respiratory area although the olfactory epithelium was completely destroyed as early as the 16th day.

DISCUSSION

In the first paper of this series (4) it was shown first, that influenza virus infection causes a complete destruction of the respiratory epithelium in the ferret's nose, and second, that during the period of repair from the 6th to 14th day after infection a strikingly abnormal type of epithelium varying from an undifferentiated low transitional type to a stratified squamous or columnar variety constitutes the respiratory epithelium. This gradually matures and differentiates until after 3 weeks a relatively normal ciliated columnar epithelium with numerous goblet cells is again seen.

In the present paper it has been found that at the time the repairing respiratory epithelium is most abnormal it is resistant not only to reinfection with influenza virus but to severe chemical stimuli which in the normal animal destroy both the respiratory and the olfactory epithelium. During the subsequent 3 to 4 weeks as the respiratory epithelium returns to normal, it again becomes susceptible to chemical injury although showing no reaction to reinfection. The first signs of loss of the refractory state appear with the return of the ciliated columnar cells.

The series of events observed, although distinctly more rapid, is quite parallel to that observed in studies of the mechanism of resistance to chemical injury which has been investigated by MacNider (6–12). This author demonstrated that the acquired immunity of an organ to functional and structural damage by a chemical poison could be correlated with an altered morphological appearance and functional response of the cells of that organ. MacNider found that immunity to uranium poisoning in dogs following a single large dose of uranium nitrate was correlated with repair of the liver and kidneys and replacement of the normal hepatic and renal tubule cells by others less specialized in appearance. Repair, which was accomplished by regeneration of cells similar in appearance to normal hepatic and renal cells and which usually occurred after a small dose of the toxic agent, failed to confer any resistance to a second dose of the poison. The resistant cells were elongated and flattened with

deeply staining nuclei and a tendency to fuse together to form syn-cytial strands. Senile but otherwise normal dogs might possess an inherent immunity to uranium and the liver of such animals showed irregular cords of flattened cells like those seen in younger dogs after the development of acquired resistance to uranium. MacNider also showed that resistance acquired as a result of damage by a chemical agent was not specific for that agent, but in the case of the liver recovery from uranium poisoning was followed by resistance to chloroform poisoning. In the case of the kidney acquired resistance to mercury bichloride poisoning was accompanied by resistance to uranium.

Much experimental work has been done on the regeneration of the nasal mucosa after injury. Boling (3) showed that during the repair process in the nasal mucosa there was a definite cycle of changes and that at one stage an abnormal stratified and many layered epithelium was present which was resistant to the necrotizing effect of zinc iontophoresis.

Whatever may be the nature of the resistance of the nasal membrane of the convalescent ferret to ionization, the demonstration that such a resistance exists is proof that the repairing epithelium differs from the normal both morphologically and functionally. This altered reaction is effective not only against the original infectious agent but against non-specific physicochemical injury as well. The return of the normal columnar ciliated cells is accompanied by a return of susceptibility to chemical injury although even 1 month after the attack of influenza there is a less complete susceptibility than in the normal ferret.

It is difficult to determine what relation the resistance herein described bears to immunity to virus infection. Both the morpho-logical abnormality of the epithelium and the functional resistance to severe chemical injury are much more short lived than the resistance to second virus inoculation. In the latter instance circulating anti-bodies represent an uncontrolled factor which makes it impossible to measure the duration of tissue resistance alone, while in the case of ionization this factor is eliminated. On the basis of the present experiments, despite the factor of circulating antibodies, it seems not unreasonable to believe that in the first 2 or 3 weeks of convalescence

after an initial attack of influenza, the respiratory tissues possess a refractory state wholly independent of specific antibodies. Subsequently it seems likely that a balance between tissue reactivity and specific antibodies comes into play. This concept will be discussed more fully in the following paper.

It may be of interest in passing to suggest that reactions of the type herein described play some rôle in the type of resistance described as "the interference phenomenon." Moreover, procedures such as resistance to chemical injury may possibly be of value in measuring variations in the susceptibility of specific cells to different infectious agents.

SUMMARY

Because of the marked morphological abnormality of the nasal respiratory epithelium in ferrets recovering from epidemic influenza virus infection, attempts were made to determine whether the anatomical changes were associated with functional changes in the epithelial cells. It was found that on the 7th or 8th day after infection, at which time an immature transitional type of epithelium covers the respiratory area, the cells are resistant not only to reinfection with influenza virus but to a severe physicochemical stimulus supplied by iontophoresis or prolonged irrigation with zinc sulfate. Later, as the ciliated columnar cells return, susceptibility to physicochemical injury returns although resistance to influenza virus persists. The ciliated columnar cells are the ones which are damaged by the physicochemical agent while the deeper cells in the regenerating area remain unaffected. 5 weeks after infection the epithelium is anatomically normal but tissue resistance to zinc sulfate is still present to some degree as evidenced by foci of undamaged cells remaining after ionization.

The olfactory epithelium which is undamaged by the PR8 strain of epidemic influenza virus also becomes resistant to ionization after infection. As soon as the respiratory epithelium exhibits any loss of resistance to zinc sulfate the chemical produces complete necrosis of the olfactory area.

The refractory state to physicochemical agents exhibited by the regenerating nasal mucosa of the ferret after influenza virus infection

is thought to be a non-specific resistant state, significant for a time at least, in the mechanism of immunity to influenza virus.

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EXPLANATION OF PLATES

All sections stained with hematoxylin and eosin.

PLATE 40

FIG. 1. Normal ferret subjected to intranasal zinc ionization and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelium has been desquamated, the submucosa is wider than normal, and an exudate is present in the air passages.

FIG. 2. High power view of Fig. 1. × 570. The epithelium to the left is composed of a flattened layer of cells incorporated with the basement membrane. The submucosa is edematous with congested vessels and polymorphonuclear leucocytic infiltration. The exudate contains leucocytes and cell debris.

FIG. 3. Convalescent influenza ferret 11-01 reinoculated with virus on the 8th day after the first infection. Sacrificed 3 days later. Anterior turbinate. × 80. The epithelium is of the stratified columnar type, and there is no sign of necrosis since the second inoculation. The exudate in the air passages is structureless.

FIG. 4. High power view of Fig. 3. × 570. The epithelium, here cut obliquely, is regular, stratified columnar and ciliated. There is no sign of recent necrosis.

FIG. 5. Convalescent influenza ferret 10-99 sacrificed on the 8th day. Anterior turbinate. × 80. The epithelium is partly stratified transitional and partly stratified columnar.

FIG. 6. High power view of Fig. 5. × 570. The epithelium is stratified and transitional in type. The stage of regeneration is clearly earlier than in ferret 11-01.
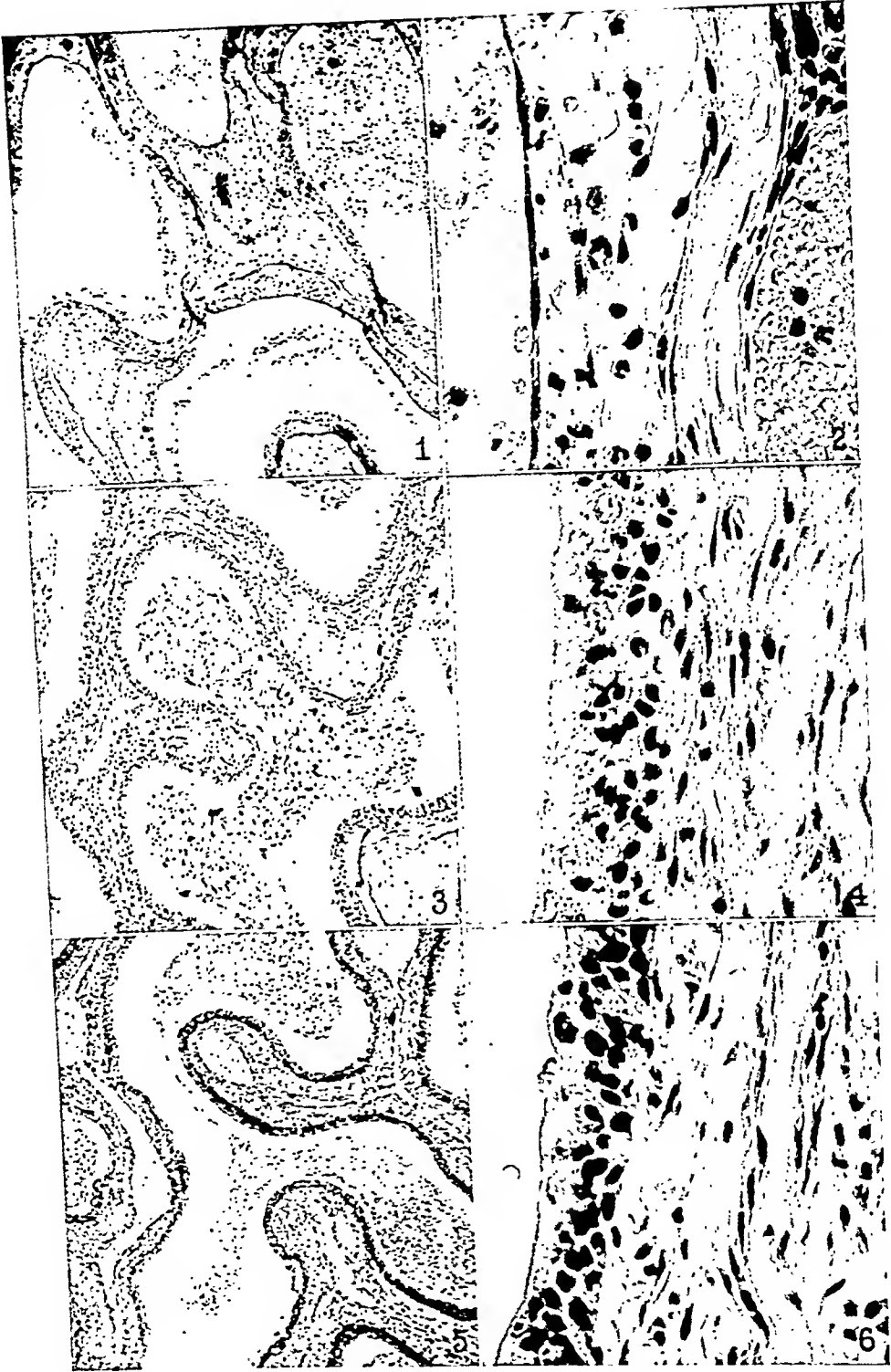
PLATE 40

(Stuart-Harris and Francis: Nasal histology of influenza infection. II)

PLATE 41

FIG. 7. Convalescent influenza ferret 10-95 subjected to intranasal zinc ionization on the 8th day and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelium is comparable with that in 10-99 and of stratified transitional or early columnar type. There is no sign of recent necrosis since the ionization. There is a little mucus in the air passages. The olfactory epithelium of this ferret was normal in appearance.

FIG. 8. High power view of Fig. 7. × 570. The epithelium is stratified columnar in type and shows leucocytic infiltration. There is no sign of necrosis since the ionization, however, and the submucosa shows no congestion.

FIG. 9. Convalescent influenza ferret 10-98 sacrificed on the 15th day. Anterior turbinate. × 80. The epithelium is regular, partly simple ciliated columnar, and partly stratified ciliated columnar.

FIG. 10. Convalescent influenza ferret 10-94 subjected to intranasal zinc ionization on the 15th day and sacrificed 24 hours later. Anterior turbinate. × 80. There has been a partial desquamation of the epithelium which varies from a narrow layer 2 cells deep to a partly ciliated layer in which some of the cells have been removed. The olfactory epithelium of this ferret was completely desquamated.

FIG. 11. High power view of Fig. 10. × 570. The epithelium is partly destroyed but the basal cells are still present and toward the bottom of the figure some ciliated cells which have resisted destruction are seen.

FIG. 12. Convalescent influenza ferret 11-11 subjected to intranasal zinc ionization on the 28th day and sacrificed 24 hours later. Anterior turbinate. × 80. The epithelial destruction is almost as severe as in a normal ferret after ionization. Note the exudate and inflammatory reaction. An area of undestroyed ciliated columnar cells is visible toward the right hand corner of the figure, and there were several areas of such epithelium in this turbinate. The olfactory epithelium was completely destroyed.

(Stuart-Harris and Francis: Nasal histology of influenza infection. II)

STUDIES ON THE NASAL HISTOLOGY OF EPIDEMIC INFLUENZA VIRUS INFECTION IN THE FERRET

III. Histological and Serological Observations on Ferrets Receiving Repeated Inoculations of Epidemic Influenza Virus

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PLATE 42

After a single intranasal inoculation of influenza virus, the ferret becomes for a time solidly immune to reinfection and shows no clinical signs of illness when reinoculated. Accompanying this immunity, neutralizing antibodies develop in the blood and, as has been shown previously (1, 2), morphological changes occur in the infected tissues so that for a time the epithelium which covers the nasal mucosa is abnormal both structurally and functionally. It seems probable on the basis of the foregoing observations that the tissue changes which occur during repair of the nasal mucosa may of themselves confer some protection against an infection which inflicts its primary injury upon the respiratory tract. In this case there may exist a short-lived refractory state of the nasal mucous membrane which subsequently is reinforced by the humoral changes. With this possibility in mind, a study was made of ferrets which had received repeated inoculations of influenza virus.

Materials and Methods

Estimations of the antibody content of the blood were made from time to time by the method of Francis and Magill (3) with the observation period prolonged to 10 days. The ferrets were killed at a time when the titer of circulating anti-

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bodies was known, and sections were prepared from the nasal fossae of the ferrets in the manner previously described (1). In this way it was hoped to correlate the histological findings in the ferret's nasal mucosa with the clinical reaction to a final test dose of virus intranasally at a time when the content of circulating antibodies was known. Some of the ferrets received subcutaneous injections of virus as well as intranasal ones, but no attempt was made to study the reaction of the nasal mucosa in ferrets which received only vaccinating doses of virus.

One group comprised 24 ferrets which, after varying numbers of intranasal or subcutaneous inoculations of virus at previous times, were given a final intranasal inoculation, observed clinically for 2 or 3 days, and then sacrificed so that histological preparations of the nasal mucosa could be obtained. A second group of 11 ferrets were subjected to repeated inoculations in a manner similar to that employed with the preceding ferrets but were sacrificed without a final intranasal test. Sections from these animals furnished histological material which served to control the findings in the first series.

The PR8 ferret passage strain of human influenza virus was used for most of the inoculations including all the final tests, but other strains of human influenza virus were used from time to time. All intranasal inoculations were given under ether anesthesia.

Histological Findings in Control Ferrets

Table I summarizes the histories of the control ferrets. It will be seen that all received one or more intranasal inoculations and 5 had received subcutaneous inoculations in addition during a period of observation varying from 6 weeks to 10 months in duration. Only 1 ferret (9-56) suffered from more than one clinical attack of influenza during this time. The ferrets were sacrificed at periods varying from 14 days to 4 months after their last intranasal inoculation of virus. As this final inoculation was not always accompanied by clinical signs of infection, the table also includes the interval from the last inoculation followed by a clinical response to the day of autopsy. None of the ferrets showed any gross abnormality of lungs or turbinates when sacrificed.

Two ferrets were sacrificed on the 14th day, 1 on the 34th day, and the rest 4 months after the last inoculation of virus which had elicited a clinical response. The histological appearance of the turbinates differed from that of normal ferrets in certain particulars. The 2 ferrets sacrificed 14 days after an attack of influenza showed the characteristic features of epithelium and cartilage regeneration seen after influenza virus infection described in the first paper of this series (1). One of them (10-53) exhibited more advanced epithelial repair than is usually observed at this stage of convalescence, the epithelium having already reached a columnar ciliated stage although areas of flattened pseudostratified epithelium covered the tip of the anterior turbinates. The second (10-57) showed the hyperplastic

TABLE I

Histological and Serological Findings in Previously Infected Ferrets Not Reinoculated at Time of Autopsy

Ferret No.	Total period of observation	Total number of intra-nasal inoculations of virus	Interval from last inoculation to day of autopsy	Number of clinical attacks	Interval from last clinical attack to day of autopsy	Titer of neutralizing antibodies	Histology of turbinates
10-53*	6 wks.	1	14 days	1	14 days	—	Epithelial irregularities; pseudostratified and stratified columnar epithelium. New cartilage
10-57	6 "	2	14 "	1	14 "	—	Epithelial irregularities; pseudostratified and stratified columnar epithelium
10-45*	2 mos.	1	34 "	1	34 "	—	Epithelial irregularities. Lymph follicle in gland
9-56	7 "	2	4 mos.	2	4 mos.	950	Normal epithelium
10-27	4 "	2	3½ "	1	4 "	35	Pseudostratified epithelium at tip
10-37	4 "	2	3½ "	1	4 "	35	Epithelium normal. Dilated venous sinuses. Lymph follicle in gland
9-57	7 "	3	3½ "	1	4 "	150	Pseudostratified epithelium at tip
9-08	10 "	3	3½ "	0	Subclinical infection	400	Pseudostratified epithelium at tip. Dilated venous sinuses
10-07*	4 "	1	4 "	1	4 mos.	100	Normal epithelium
9-46†	7 "	2	4 "	1	4 "	400	Chronic changes: fibrosis, squamous and stratified columnar epithelium
7-75†	10 "	2	4 "	1	4 "	280	Pseudostratified epithelium at tip. Scanty exudate

* Received virus subcutaneously prior to first intranasal inoculation.

† Received virus subcutaneously between first and second intranasal inoculations.

stratified columnar ciliated epithelium which is usually found on the 14th day after an attack of influenza.

In all but one of the ferrets which were killed at later periods after their last clinical response to inoculation, the turbinates were normal in the gross. However, microscopically these showed the pseudostratified epithelium at the tip of the anterior turbinates and the slight increase in fibrous tissue in the submucosa which have been found to persist for some months after an attack of influenza. The turbinates of 2 ferrets (10-37 and 9-08) showed a dilatation of the venous sinuses, 2 (10-37 and 10-45) exhibited a well developed lymph node in the submucosa of the lateral nasal gland, and 1 (7-75) showed a normal epithelium but contained an excess of mucus with polymorphonuclear leucocytes in one portion of the air passages. The last of this group of control ferrets (9-46) presented a definitely abnormal structure of the turbinates. On one side of the nose the mucous membrane of the turbinates was fibrotic; the epithelium was hyperplastic and of stratified columnar ciliated type. On the other side the turbinate tissue was normal except for three laminae which were fibrotic, showed areas of hemorrhage into the submucosa, and were lined by a low flattened squamous epithelium. As this ferret had not had an inoculation for 4 months previously, it is apparent that chronic changes can persist in the turbinates for long periods of time after influenza, possibly as a result of a superadded bacterial infection.

In general, among these control ferrets the nasal mucosa was found to have returned to normal in spite of repeated intranasal inoculations of virus even when these were accompanied by clinical attacks of influenza. Certain features, such as a pseudostratified arrangement of the epithelium at the tip of the nose, irregularities of the venous sinuses, increase in fibrous tissue of the submucosa, and lymphocytic infiltration of the lateral nasal gland, suggested residual abnormalities of infection and regeneration in these ferrets. Some of them would undoubtedly have been clinically responsive to intranasal influenza virus at the time of sacrifice but others would probably have been immune. The essential fact, however, is that in most instances it has been impossible to demonstrate without further manipulation any simple histological alteration which differentiates the animal completely recovered from repeated inoculations of epidemic influenza virus from ferrets which have never been subjected to infection. Repeated stimuli have not resulted in a permanent anatomical change in the epithelium of the respiratory mucous membrane approaching that seen temporarily in the period of repair from primary infection.

Histological Findings in Ferrets Recently Reinoculated

The animals in this group of 24 ferrets were reinoculated 2, 3, or 4 days before sacrifice and according to their clinical response may be classified into three groups: (a) those which developed undoubted clinical signs of illness after the test inoculation; (b) those which were clinically immune in that no fever, nasal discharge, or other signs of infection were detected; and (c) those which developed doubtful signs of illness such as fever only or symptoms only.

Ferrets Responsive Clinically to the Test Inoculation.—The first group includes 9 ferrets all of which responded to the test inoculation of virus with signs of illness of varying severity as listed in Table II. In each case the final illness represented the second clinical attack of influenza virus infection although 1 ferret had received two previous intranasal inoculations of virus. It will also be seen that 1 ferret received a subcutaneous inoculation of virus, but the two clinical attacks of influenza occurred at a later date than the vaccination so that the final histological findings were probably not influenced by the subcutaneous inoculation. Intervals of from 3 to 14 months had elapsed between the two clinical attacks of influenza in the various ferrets but here again the histological findings bore no clear relation to the length of the interval between attacks. The majority of the ferrets were sacrificed on the 3rd day after inoculation but 2 were sacrificed on the 2nd and 1 on the 4th day after inoculation.

Table II also shows the clinical features of the attack of influenza induced by the test dose of virus. All of the ferrets showed fever, all developed nasal signs, such as nasal discharge and sneezing, but only 2 ferrets showed definite abnormalities of respiration. At post mortem the modification of infection as measured by pulmonary involvement in these ferrets compared with that seen in normal ferrets infected for the first time was obvious. The lungs of 5 of the ferrets were normal, the lungs of 3 others showed mottled areas without true consolidation, and only 1 ferret showed the typical lung lesions of influenza virus infection. Yet with this particular strain of virus, inoculation under an anesthetic invariably caused the production of extensive lung lesions in normal ferrets. There was no doubt, therefore, that the infection was modified clinically in these ferrets during their second attack of influenza.

With regard to the nasal reaction, the turbinates macroscopically appeared glistening and injected as in the case of turbinates during a first attack of influenza. One ferret (10-05) exhibited frank pus in the nasal passages but in the others only a watery exudate was observed.

Microscopically the turbinates from 3 ferrets showed lesions typical of those seen during a first attack of influenza virus infection between the 2nd and 4th days of illness. There was epithelial necrosis and desquamation; the submucosa was infiltrated with polymorphonuclear leucocytes and showed edema and congestion; and the air passages were occupied by an exudate of mucus, debris, and leucocytes. The epithelium in the respiratory area consisted of a single layer of basal cells which was closely incorporated with the basement membrane.

TABLE II

Histological and Serological Findings in Previously Infected Ferrets Again Clinically Responsive to Test Inoculation

Ferret No.	Total period of observation	Total number of intranasal inoculations	Number of clinical attacks	Interval between attacks	Severity of last clinical attack			Interval from last inoculation to day of autopsy	Titer of neutralizing antibodies at time of test inoculation	Histology of turbinates
					Fever	Nasal symptoms	Respiratory symptoms			
9-66	5 mos.	2	2	5 mos.	++	±	0	3 days	+ 5 mos. before	Necrosis, foci of normal epithelium, accelerated repair
9-67	5 "	2	2	5 "	++	++	0	3 "	+ 5 " "	Necrosis without repair
9-69	6 "	2	2	5 "	++	++	±	3 "	<50	Necrosis, foci of normal epithelium, accelerated repair
9-98	4 "	2	2	4 "	++	++	++	3 "	0	Necrosis, accelerated repair
8-28	14 "	2	2	14 "	++	±	++	2 "	2	Necrosis, foci of normal epithelium, accelerated repair
8-35	14 "	2	2	14 "	++	++	0	2 "	150	Necrosis, foci of normal epithelium, accelerated repair
8-39	14 "	2	2	14 "	++	++	±	4 "	100	Necrosis without repair
10-25	4 "	3	2	4 "	++	++	0	3 "	18	Necrosis, accelerated repair
10-05*	3 "	2	2	3 "	++	±	0	3 "	—	Necrosis, accelerated repair; residual foci of polygonal cells; Necrosis without repair; some foci of normal epithelium

* Received virus subcutaneously prior to first intranasal inoculation.

The turbinates of the 6 other ferrets which exhibited clinical evidence of infection showed significant differences from those seen in the acute stage of a first attack of influenza. Epithelial necrosis and desquamation were present in all 6 turbinates but the residual respiratory epithelium instead of being a flattened pavement layer was in many places two or three cells deep (Figs. 1 and 2), thus resembling the transitional epithelium seen on about the 6th to 8th days of a first attack of influenza. Moreover, the epithelial necrosis was not uniform, and in 1 ferret (9-66) there were in the anterior turbinates extensive areas of unaffected ciliated columnar epithelium immediately adjacent to areas of necrosis (compare Figs. 3 and 4 from ferret 9-47). The submucosa showed a richer infiltration of cells than ordinarily observed even in a first attack although mononuclear cells preponderated over the polymorphonuclear leucocytes. It was evident, therefore, that the cellular reaction to the virus in these latter ferrets was sharply different from that in previously uninfected normal ferrets.

The epithelial reaction is of particular interest since at the time the respiratory area of the nasal mucous membrane of a previously untreated ferret would be stripped to the basement membrane, in the present animals a multilayered epithelium was observed. Moreover, frequent areas of relatively normal epithelium had escaped damage. Two possible explanations for the presence of multilayered epithelium as early as the 3rd day after infection are suggested. The first is based upon the observations reported in the preceding paper of this series (2) that in the regenerating epithelium a return to susceptibility to chemical injury is paralleled by the return of the normal ciliated columnar cell. Hence if only the superficial columnar cells were susceptible to repeated virus infection the underlying cells would remain undamaged. This explanation would of necessity indicate that the respiratory epithelium of the ferret before reinfection is stratified columnar in type. The appearance of the turbinates in 1 animal (10-25, Figs. 5 and 6) in which exceptionally well developed areas of stratified cells were present on the 3rd day after reinoculation lends support to this view. Furthermore, it has previously been observed (1) that pockets of stratified epithelium may persist after a primary infection. On the other hand, in control animals of the present series, in repeatedly inoculated ferrets no suggestion of persistence of a widespread stratification of cells in the respiratory epithelium was found.

The other explanation suggested for the presence of a many

layered epithelium by the 3rd day after infection is that an acceleration of the repair process occurs in animals receiving repeated injuries. In the first place, in these animals the basal layer of cells was cubical or polygonal in shape with large nuclei. Such cells were found even in the basement membrane of the turbinates of the 3 ferrets described above in which the appearance was otherwise similar to that in a first attack of influenza. In the second place, numerous mitoses were present in the more superficial cells and the appearance of the whole epithelium suggested an area of active regeneration. Furthermore, the stratified form of epithelium was not observed in the control animals not subjected to reinoculations of virus, while in at least 6 of the 9 ferrets examined during the acute stage of a second attack of influenza the residual epithelium was distinctly of the transitional type. The evidence suggests then that as a result of infection with epidemic influenza virus a conditioning of the basal cells of the epithelium occurs, so that when a sufficient decrease in immunity permits the induction of a second clinical attack the whole process of repair is greatly accelerated.

Ferrets Immune to the Test Inoculation.—11 ferrets showed no clinical signs of illness after the final inoculation (Table III). They were considered clinically immune and were sacrificed on the 3rd day after inoculation in order that histological preparations could be obtained from their nasal turbinates. At post mortem the lungs of 7 of the ferrets were normal, 1 showed lesions atypical of influenza, and 3 showed lesions suggesting healing influenza virus lesions. Macroscopically the turbinates were normal in 8 ferrets, but in 2 ferrets pus was present, and in 1 other the inferior portion of the anterior turbinate was dark red and hemorrhagic in appearance. Microscopically none of the turbinates showed lesions suggesting a recent necrosis such as might have been induced by the test inoculation of virus.

In the group were 5 ferrets sacrificed 3 days after the last test dose of virus but 3 weeks after the previous dose which elicited clinical infection. All of these animals had been given virus subcutaneously prior to the first intranasal inoculation. The turbinates of 4 of them showed epithelial irregularities and cartilage regeneration typical of the 3rd week of repair after an original infection. The other (10-44) presented an extraordinary degree of fibrosis of the turbinates together with a hyperplastic stratified columnar epithelium and leucocytic infiltration. Frank pus was detected in the nasal passages of the latter animal at post mortem, and the histological picture was thought to represent the result of a superadded bacterial infection secondary to the influenza virus infection 3 weeks previously.

TABLE III

Histological and Serological Findings in Previously Infected Ferrets Clinically Immune to Test Inoculation

Ferret No.	Total period of observation	Total number of intranasal inoculations	Number of clinical attacks	Interval from last attack to day of autopsy	Interval from last test to day of autopsy	Gross appearance at autopsy		Titer of neutralizing antibodies at time of test inoculation	Histology of turbinates
						Lung	Nose		
9-11	9 mos.	3	1	9 mos.	3 days	Normal	Normal	400	Pseudostratified epithelium at tip
9-53	7 "	4	1	4 "	3 "	"	Inferior part dark red	280	Normal but for 3 laminae with transitional epithelium and leucocytic infiltration
9-03	9 "	4	2	3 "	3 "	"	Normal	—	Pseudostratified epithelium at tip; fibrosis of submucosa; new cartilage
9-60	6 "	4	1	3 "	3 "	"	"	—	Normal but for fibrosis of a few laminae with squamous epithelium and leucocytic infiltration
6-15	18 "	4	2	15 "	3 "	Atypical in left upper lobe; Old lesion	Pus in nasal passages	400	Normal but for epithelial cysts and leucocytes
9-15*	7 "	3	1	4 "	3 "	Normal	Normal	150	Epithelial irregularities; lymphocytic infiltration of gland
10-13†	6 wks.	2	1	28 days	3 "	Old lesion	Pus in nasal passages	400	Slight epithelial irregularity; lymph follicle in gland
10-14†	6 "	2	1	21 "	3 "	Normal	Normal	400	Fibrosis; stratified columnar epithelium; leucocytic infiltration; new cartilage
10-16†	6 "	2	1	21 "	3 "	"	"	500	Normal epithelium; new cartilage
10-50†	6 "	2	1	21 "	3 "	"	"	960	Pseudostratified epithelium at tip; new cartilage
10-52†	6 "	2	1	21	3 "	"	"	1400	Epithelial irregularities; new cartilage

* Received virus subcutaneously between first and second intranasal inoculations.

† Received virus subcutaneously prior to first intranasal inoculation.

One other ferret received virus subcutaneously in the interval between the fir and second intranasal inoculations. The nasal mucosa of this animal (9-45) was normal.

The remaining 5 ferrets received only intranasal inoculations of virus, the last being 3 days before death, and they form a group comparable with the control ferrets. 2 of these ferrets (9-11 and 9-03) exhibited no abnormalities in the tissues except for the presence of pseudostratified epithelium at the tip of the nose and a slight increase in fibrous tissue in the submucosa. One ferret (6-45) showed a normal nasal mucosa except for a small area with cysts lined by columnar epithelium and containing leucocytes. Frank pus had been found in the nasal passages of this ferret at post mortem. The remaining 2 ferrets (9-53 and 9-60) showed abnormalities of the turbinates resembling those seen in ferret 9-46 of the uninoculated control series. Some of the laminae were fibrotic, infiltrated with red blood cells, and covered by a low flattened squamous epithelium with leucocytic infiltration. The only feature suggesting that these lesions were different etiologically from those seen in ferret 9-46 and that they were due to the recent inoculation of virus was the leucocytic infiltration of the epithelium.

Summarizing the findings among ferrets which were clinically immune to their final test dose of virus, none showed definite lesions of the nasal mucosa resembling recent virus lesions. Some of the ferrets showed an abnormal appearance of the turbinates and the possibility that these abnormalities represented focal necrotic lesions of virus etiology could not be excluded. However, the fact that 1 of the control ferrets not recently inoculated had shown somewhat similar lesions was thought to indicate that the abnormalities in the recently inoculated immune ferrets were due to chronic changes, possibly the result of superadded bacterial infection.

Ferrets with Doubtful Clinical Reaction to the Test Inoculation.—Finally, there were 4 ferrets in which clinical signs of doubtful significance developed following the final reinoculation. 2 (9-47 and 10-06) developed nasal symptoms without elevation of temperature, and 2 (9-59 and 7-51) developed a rise of temperature without nasal symptoms or other signs of illness. At autopsy the lungs of both 9-47 and 10-06 showed small lesions which were atypical of influenza virus lesions in appearance, and the lungs of 9-59 and 7-51 were normal. 3 of the ferrets, as shown in Table IV, had received a subcutaneous inoculation of virus at some time in the past. The histological findings suggested that ferrets 9-47 and 10-06 were undergoing reinfection of the turbinates by influenza virus but that 9-59 and 7-51 were not. Thus the turbinates of 9-47 and 10-06 exhibited widespread epithelial necrosis with desquamation, accelerated repair of the basal layer of cells, and es-

cape of some areas of ciliated epithelium adjacent to areas of necrosis (Figs. 3 and 4 from ferret 9-47). The turbinates of 9-59 and 7-51, on the other hand, showed an absence of epithelial necrosis and were for the most part normal. However, a few of the laminae of 9-59 were fibrotic and covered by a squamous epithelium as in the case of some of the clinically immune ferrets described above (9-53 and 9-60). Nearly all of the turbinate epithelium was normal in 7-51 but there was a

TABLE IV

Histological and Serological Findings in Previously Infected Ferrets with Doubtful Clinical Reaction to Test Inoculation

Ferret No.	Total period of observation	Total number of intra- nasal inocu- lations	Interval from last attack to day of autopsy	Interval from last test to day of autopsy	Reaction to test inocula- tion			Titer of neutralizing an- tibodies at time of test inoculation	Histology of turbinates
					Fever	Nasal symp- toms	Respiratory symptoms		
9-47*	7 mos.	3	4 mos.	3 days	0	+	0	100	Necrosis, foci of normal epithelium; acceler- ated repair
10-06†	4 "	2	4 "	3 "	0	+	0	100	Necrosis, foci of normal epithelium; acceler- ated repair
9-59	7 "	4	7 "	3 "	+	0	0	150	Normal but for fibrosis of a few laminae with squamous epithelium and leucocytic infiltra- tion
7-51*	11 "	3	4 "	3 "	+	0	0	400	One small area of transi- tional epithelium and leucocytic infiltration

* Received virus subcutaneously between the first and second intranasal inocu- lations.

† Received virus subcutaneously prior to the first intranasal inoculation.

tiny focus of transitional type of epithelium infiltrated with leucocytes. It is possible, therefore, that the abnormalities in the turbinates of these 2 ferrets represented focal lesions.

The Relation between Antibody Titer and Resistance

Histological studies in animals which were completely immune to reinfection several months after one or more previous inoculations

with epidemic influenza virus have yielded no evidence that a new type of resistant epithelium develops in the nasal respiratory area as a result of repeated inoculations with epidemic influenza virus. Abnormalities considered to be reflective of earlier infection were seen both in the uninoculated controls and in the animals which were clinically immune at the time of the final test. In the controls, however, changes of this type were irregularly distributed both in animals which would most certainly have been resistant to test inoculation and in others which would with equal probability have been susceptible. The abnormalities observed did not involve the entire epithelium but consisted of small cyst-like areas in the epithelium, persistence of pseudostratified epithelium at the anterior tip of the turbinates, epithelial irregularities and fibrous changes in the submucosa and cartilage. It was not possible, therefore, with any confidence to correlate the immunity or susceptibility of ferrets after repeated virus inoculations with mere structural changes in the epithelium. Accordingly, titrations were made of neutralizing antibodies in the serum of 19 ferrets immediately prior to the immunity test in order to ascertain whether a relationship existed between resistance to reinfection and the level of circulating antibodies.

The animals included in the series had received previous inoculations of virus from 21 days to 15 months before the present test. Serum was obtained 3 to 5 days before the test and the titrations were done without knowledge of the clinical response or the histological evidence. Thus the clinical, the histological, and the serological results were arrived at by observers entirely independent of each other. The results are presented in Chart 1.

The vertical columns at the head of which the ferret numbers are given represent the titers of circulating antibodies. At the side of the column is shown the interval since previous inoculation with virus, and above, a brief statement of the histological findings. The letters *I* and *S* indicate that the animals were clinically immune or susceptible to the test inoculation.

On the extreme left of the chart are animals which received a fresh inoculation of virus only 3 weeks subsequent to an acute attack and which would be expected to be completely immune. They were immune, possessed the highest titers of neutralizing antibodies, and at the same time revealed no pathological changes which could not be fully accounted for by the earlier infection.

The next group to the right comprises 6 animals, which were probably clinically
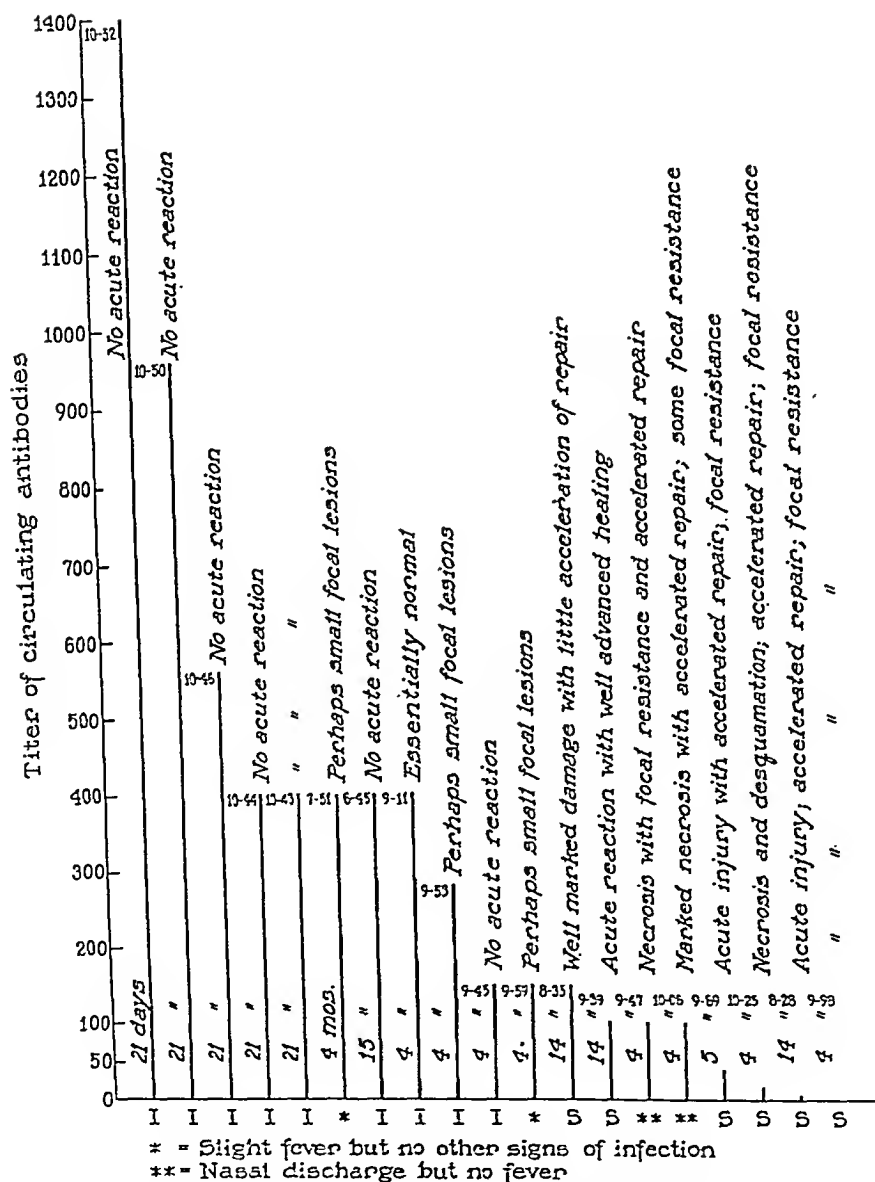
CHART 1. Relation of titer of circulating antibodies to clinical and histological reaction in reinoculated ferrets.

resistant, but in 2 of which (7-51 and 9-59) a slight, brief rise of temperature occurred without other clinical signs of infection. One had been allowed to wait 15 months since previous test, the others 4 months. Histological study of the turbinates of all 6 animals failed to reveal evidence of acute injury to the nasal epithelium although 3 of them showed small foci of abnormal epithelium possibly related to the virus inoculations.

Beyond this point a different sequence of events occurs since all of the 8 animals at the right of the chart exhibited acute nasal reactions to the reinoculation of virus. Of the 8, 3 had not received virus for 14 months, 1 for 5 months; the other 4 had been tested 4 months previously. The antibody titers varied from 1:150, a level which in 2 other ferrets was associated with immunity, to a complete absence of antibody in 1 animal. In 2 animals the clinical reaction was extremely mild; there was no rise of temperature to the ordinary febrile level but nasal discharge developed. Associated with lower antibody titers and clinical susceptibility was the fact that necrosis of the respiratory epithelium was observed in each case. In most instances the tissue damage was marked but despite the extensive necrosis of the epithelium, areas of resistant epithelium, normal in appearance, were invariably present. Furthermore, in all but one a marked acceleration of the repair process, as compared with previously untreated ferrets, was observed. This acceleration, previously mentioned, is indicated by the rapid proliferation of the basal cells of the respiratory epithelium producing by the 3rd day after virus inoculation a stage of repair equivalent to that observed on the 8th day after a primary infection.

The results clearly suggest that in ferrets 4 months or more after previous virus inoculation a parallelism tends to prevail between the height of antibody titer to the virus, the resistance of the nasal respiratory mucosa to reinfection, and the clinical response to reinoculation. It seems that the antibody titer supplies a factor for estimating resistance to reinoculation (at least, in animals repeatedly inoculated) which could not be derived from histological studies of animals before inoculation. That a level of antibody exists which separates immune from susceptible ferrets has been indicated by the observations of Smith (4). The results of studies in the present group of ferrets suggest that the dividing line occurs at a titer of about 1:100 to 1:150. The animals with titers above this level show little or no clinical evidence of infection and exhibit no significant damage to the respiratory epithelium while below this range of antibody titer nasal damage and clinical infection are the rule. In the intermediate zone variability of response occurs.

DISCUSSION

The previous papers of this series (1, 2) have presented evidence to show that influenza virus inflicts a specific injury which destroys the respiratory epithelium in the nose of the ferret. In the process of repair a modified epithelium develops which between the 6th and 8th days is of a transitional immature type. This abnormal epithelium is resistant not only to the virus causing the original damage but to ionization or irrigation with zinc sulfate, a procedure which causes extensive destruction of the entire nasal epithelium in the normal ferret. The period during which the abnormal, resistant epithelium persists is, however, transitory and after 21 days a relatively normal respiratory epithelium is encountered. At that period the tissue is again susceptible to physicochemical injury although still resistant to influenza virus. The demonstration of the nonspecific tissue resistance in an abnormal cell type indicates clearly that in the early period of convalescence resistance to reinfection may bear no relation to the immunological activity of the blood or the tissues. The resistance of the epithelium, however, as measured by its immunity to physicochemical damage is of brief duration and it has not been possible to measure the duration of only the cell refractoriness to the virus since circulating antibodies cannot be eliminated from consideration.

It seemed possible, nevertheless, that with repeated exposures to influenza virus a more permanent structural change might be induced in the respiratory mucous membrane and that the persistence of immunity might be closely related to the length of time through which the modified epithelium covered the normally vulnerable area. The observations in the present paper were made for the purpose of determining if such an alteration did exist. While certain limited changes reflected previous infection there was no evidence that as a result of repeated insults with epidemic influenza virus the respiratory mucous membrane develops a morphologically different epithelium persisting beyond the period of acute injury. In general, the histological appearance of the epithelium differs in no well defined manner from that of the normal untreated ferret.

On the other hand, there is evidence that a conditioning of the

respiratory epithelium occurs as a result of infection, perhaps accentuated by repeated inoculation of virus. This is reflected in animals which have lost sufficient immunity to render them clinically reactive to a fresh virus exposure. In these cases, while extensive necrosis of the respiratory epithelium is again produced there are considerable areas of ciliated columnar epithelium which completely escape injury. There is also a marked acceleration of the repair processes so that a well developed multilayered transitional epithelium is seen in the damaged areas as early as the 3rd day, whereas at a similar time in the animal experiencing its first attack, complete desquamation is observed without any evidence of repair. The comparable stage in the latter animals is not reached until the 6th to 8th days.

Thus, contrary to expectations, the anatomical changes do not suffice to explain a continued complete immunity. However, resort to the serological evidence has resulted in the impression that a parallelism exists between neutralizing antibody titer and resistance to reinfection as determined by clinical signs and histological studies. Above a titer of 1:150 resistance was uniformly observed, while below this level distinct evidence of infection was invariably present. Although in the early stages of convalescence a non-specific tissue immunity is associated with an abnormal type of epithelium and in animals previously infected a conditioning of the epithelium results in a greatly exaggerated reparative capacity in later infections, the conclusion seems inescapable that after a period of months and in animals receiving repeated inoculations, at least, immunity is closely associated with the presence of an effective amount of circulating antibody which tends to protect the cellular structures.

It is of interest to attempt the application of these observations to the problem of human influenza. The complete destruction of the respiratory epithelium seen early in infection creates an ideal opportunity for bacterial invasion, particularly if pathogenic organisms are already resident in the injured areas. Later in convalescence the regenerating epithelium is highly resistant to injury of all types, thus reducing the possibilities of relapses or secondary infections. By this time circulating antibodies are developed in sufficient amount to furnish an additional protective mechanism. With the passage of time and the return of the epithelium to normal the resistance of the

tissues is lost and the serological factors assume a more important rôle in the prevention of reinfection. With repeated exposures, however, the vulnerable epithelium has developed the capacity to initiate regenerative processes extremely soon after damage has occurred. Hence, antibodies prevent too extensive injury and the conditioned cells hasten the repair so that together a distinct modification of the infection results.

It seems probable that complete immunity may be a product of the interaction of these forces. Repeated inoculations probably result in a more stable antibody level than is the case after a single attack. A further acceleration of the repair processes would then result in a period of injury and symptomatology so brief as to be considered subclinical or of an entirely different nature.

The implications of the observations in the problem of resistance to infectious disease in general and to other respiratory disease, especially the common cold, are obvious. Moreover, the accelerated reaction of repair tends to substantiate the impression gained in earlier studies (5) that in animals which have lost a certain degree of immunity the response to second infection resembles an accelerated immune reaction.

SUMMARY

A study of the respiratory mucous membrane was made in the turbinates of ferrets which had received repeated inoculations of influenza virus. There was no evidence that persistent immunity is related to the presence of a structural modification of the respiratory epithelium. In fact, the respiratory epithelium in fully immune animals differs histologically only in minor respects from that of the normal, untreated ferret. On the other hand, a functional difference exists between the normal and the previously infected animals as evidenced by a marked acceleration of the repair process in the latter.

Serological studies at the time of reinfection, 4 months or more after the previous attack, indicate that a relation exists between the height of antibody titer and resistance. The degree of immunity is probably a product of serological immunity and the rate of tissue repair.

The implications of these studies to the problem of influenza in man are discussed.

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EXPLANATION OF PLATE 42

All sections stained with hematoxylin and eosin.

Fig. 1. Influenza in a previously inoculated ferret. Day 3. Ferret 9-69. Anterior turbinate. × 80. The ciliated cells of the epithelium have been desquamated; there is exudate and inflammatory reaction. The residual epithelium is several cells deep and corresponds to that seen on about the 6th day after a first attack of influenza.

Fig. 2. High power view of Fig. 1. × 570. The epithelium is of a transitional type and shows leucocytic infiltration. The basal cells are cubical in shape and the appearance suggests a regenerative process.

Fig. 3. Influenza in a previously inoculated ferret. Day 3. Ferret 9-47. Anterior turbinate. × 80. Illustrates the presence of resistant areas of epithelium. On one side of the lamina the epithelium has been desquamated to a single layer of cells and on the other side it is normal in appearance. There is exudate and inflammatory reaction.

Fig. 4. High power view of Fig. 3. × 570. The epithelium covering the lamina on one side is necrotic and desquamated (above) and on the other is unaffected (below). The submucosa is abnormal beneath the desquamated epithelium but unaffected beneath the normal epithelium.

Fig. 5. Influenza in a previously inoculated ferret. Day 3. Ferret 10-25. Anterior turbinate. × 80. Epithelial desquamation, exudative and inflammatory reactions are present but the residual epithelium varies in thickness and has pockets of stratified cells.

Fig. 6. High power view of Fig. 5. × 570. A pocket of stratified cells with some leucocytic infiltration which suggests that here an area of stratified epithelium remained after the first attack of influenza and that this was partially resistant to reinfection.

(Francis and Stuart-Harris: Nasal histology of influenza infection. III)

PLASMA PROTHROMBIN: EFFECT OF PARTIAL HEPATECTOMY*

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In previous publications, Smith, Warner, and Brinkhous (1, 2) have noted a profound fall in plasma prothrombin in dogs having chloroform or phosphorus intoxication. The fall corresponds fairly closely to the degree of liver injury, and the prothrombin returns to normal as the liver regenerates. This evidence indicates that the liver plays an essential rôle in the manufacture of prothrombin. Further support for this conclusion is obtained by experiments presented at this time, showing that partial hepatectomy in rats also causes lowering of the plasma prothrombin level. The degree of lowering is somewhat variable from rat to rat, but in many instances the prothrombin falls to very low levels during the first 24 hours after operation. The prothrombin level returns to normal in the period required for restoration of the liver to normal weight.

Methods

Albino rats were used in all cases. Throughout the experiment they were kept on a diet of dog chow.[1] Rats in three different age groups were used. The youngest rats ranged in weight from 90 to 100 gm., the next group from 150 to 200 gm., and the oldest from 250 to 400 gm. The results obtained in the three different groups were essentially the same. Under ether anesthesia the liver was exposed and the large median lobe and the left lateral lobe were removed by the technique of Higgins and Anderson (3). It is estimated that about 60 per cent of the liver was excised in these animals. In addition, the right lateral lobe was

[1] Purina Mills, St. Louis.

partially removed in a few cases, leaving intact only the Spigelian lobe and th stumps of the other lobes. In these cases only about 25 per cent of the liver wa left behind. Prothrombin analyses were made by the method developed in this laboratory (1, 2). Blood for the analyses was drawn from the jugular vein into a 1 cc. syringe containing isotonic potassium oxalate. To avoid making the rats anemic, the individual animals were bled infrequently, but at intervals designed to cover the entire recovery period. Fibrinogen determinations were made according to the technique of Jones and Smith (4) on a number of the rats post-operatively.

EXPERIMENTAL RESULTS

The postoperative prothrombin values obtained in 39 rats are shown in Table I. The prothrombin values are tabulated in per cent of normal. In the first 9 rats shown in this table, approximately 75 per cent of the liver was removed; in the other experiments only 60 per cent. In both groups the prothrombin commonly falls to 30 to 40 per cent of normal within the first 24 hours. However, with the more drastic operation the level sometimes falls to less than 30 per cent, and in the milder operation many of the animals maintained levels of 70 per cent or more. On removal of 75 per cent of the liver, a number of animals died within 24 hours after operation. Autopsy in this group occasionally showed massive hemorrhage into the peritoneal cavity or into the tissues adjacent to the incision. Evidently, removal of this amount of liver at times permits the plasma prothrombin to fall to critical levels. No evidence of excessive hemorrhage was encountered in the animals in which only 60 per cent of the liver was removed.

The fall in prothrombin appears to reach its maximum within 24 hours. Blood samples following removal of liver tissue drawn within the first hour showed little or no fall. At the end of 6 hours the fall is often maximal. No doubt the formation of fibrinous exudate at the site of operation increases the consumption of prothrombin beyond normal limits, thus accelerating the decline. The normal intact liver is able to meet such demands, for in several control experiments simple laparotomy was performed and after some trauma to the peritoneal surfaces the incision was sutured in the usual way. The amount of exudate formed was almost as much as in the ablation experiments, yet the prothrombin showed no fall whatever. In several control experiments pieces of fresh rat liver were placed in the

TABLE I

Plasma Prothrombin Following Partial Hepatectomy

Rat No.	Prothrombin (per cent of normal) at intervals following hepatectomy							
	20–60 min.	6–7 hrs.	1 day	2–3 days	4–6 days	8–13 days	14–17 days	18–32 days
	75 per cent of liver removed							
1			17					
2			27					
3			36					
4			37	65		96		
5			39					
6			46			84	83	
7			63		55	77		123
8			63		74	85		100
9			65		62	95		81
	60 per cent of liver removed							
10		30						
11		34	32	36				
12		37	33	42	73			89
13		38	33	30	67			93
14			35					102
15			36					
16	65		37					
17				46			85	97
18					58			98
19				50		84	97	
20			41		65			
21	104		42		83	94		
22	100		46		72	83		
23			51					
24			56		113		100	
25			60					
26			63		84		102	
27	66							
28			66					
29			66					97
30			71		67			100
31			75					96
32			80					
33			84		73			105
34			85					
35			88					
36					88			
37						88		
38							100	
39			100					

peritoneal cavity to serve as a control for the autolyzed bits of liver tissue left at the stumps of resected lobes in the hepatectomized animals. These animals likewise showed no fall in plasma prothrombin at any time.

The return of prothrombin toward normal levels is manifest within 3 to 6 days, but as a rule is not complete until 10 days to 3 weeks have elapsed. Exudate formation subsides long before the prothrombin reaches normal values. The subnormal values are thus to be correlated with defective production of prothrombin, not with excessive utilization. It cannot be denied that trauma to the residual liver may decrease its functional capacity for a period of several days. We believe, however, that the actual loss of liver substance is mainly responsible for the deficient production of prothrombin. Our experiments are in accord with the work of Higgins and Anderson (3) which showed that 10 days to 3 weeks must elapse before the liver has returned completely to normal size. This period corresponds with the period of subnormal prothrombin production.

These liver ablation experiments support our work on chloroform intoxication (1, 2). In both cases an observable defect in liver tissue is associated with a marked fall in plasma prothrombin. It is conceivable that chloroform may injure other organs, though all investigators agree that the liver lesions are the only conspicuous lesions. The ablation experiments offer no injury to other organs. It is thus necessary to conclude that the liver is vitally concerned in the manufacture of prothrombin.

The fibrinogen values obtained on a number of rats postoperatively were all normal or above normal This indicates that the reduction in liver function, although sufficient to result in a decrease in prothrombin, was not sufficient to reduce the fibrinogen. Evidently the reserve capacity for prothrombin manufacture is less than in the case of fibrinogen. This is in accord with experiments from this laboratory (2) which show that mild chloroform injury in dogs produces a fall in plasma prothrombin, but not in plasma fibrinogen.

SUMMARY

Extirpation of a large portion of the liver in rats results in a marked decrease in the plasma prothrombin. The plasma prothrombin level

gradually returns to normal during the period required for restoration of the liver to its normal weight. The decrease in prothrombin incident to partial hepatectomy supports the thesis that the liver is concerned in the manufacture of plasma prothrombin.

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CELLULAR REACTIONS TO TUBERCULO-PROTEINS COMPARED WITH THE REACTIONS TO TUBERCULO-LIPIDS

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We have been studying the cellular reactions to different preparations of tuberculo-protein. In accordance with the plans of the Research Committee of the National Tuberculosis Association, of which Dr. William Charles White is Chairman, all of the tubercle bacilli used for this study were grown on a liquid synthetic medium entirely free from protein. In this way none of the preparations was contaminated with traces of any other protein. Since the work of Goldmann (1, 2), it has been known that materials injected into the tissues as particulate matter are engulfed by the phagocytic mononuclear cells. The tuberculo-proteins offer material with which to compare the effect of two substances which are certainly nearly the same except that one can be introduced in solution and the other in suspension.

The preparations of the proteins obtained from the media were given to us by Dr. Florence B. Seibert, of The Henry Phipps Institute for the Study of Tuberculosis, Philadelphia, and by Dr. John Reichel, of Sharp and Dohme, Glenolden, Pa. Drs. Seibert and Munday gave us some of their most highly purified protein, designated TPA (3), and at our request a preparation of the relatively insoluble protein, H-1. Dr. Reichel gave us the protein MA-100 which was obtained by precipitation with magnesium sulfate. To Drs. Michael Heidelberger and Arthur E. O. Menzel, of the Presbyterian Hospital, we are indebted for the proteins from the bacilli. They found (4) that different preparations of protein could be obtained from the bacilli at different pH values of the extractives. The two preparations used for this study were designated by them G and K. The G fraction

837

was obtained with water made alkaline to about pH 11.0 after previous extraction with less alkaline buffers. These processes were carried out in the cold. The material K was obtained from the residual material after extraction of the protein G by means of 0.1 N sodium hydroxide solution at room temperature. All the proteins used were from the human strain, H-37.

Studies employing the method of tissue culture by Lewis and Lewis (5), by Lewis (6), and by Carrel and Ebeling (7) have demonstrated that with appropriate culture media the monocyte of the blood stream becomes a cell which looks exactly like the macrophage or clasmatocyte of the tissues. The terms macrophage and clasmatocyte are interchangeable, each referring to different properties of the same cell. The name macrophage was introduced by Metchnikoff and means "big eater," signifying phagocytic function: the term clasmatocyte was introduced by Ranvier and refers to the property of desquamation of the surface film, a phenomenon which characterizes this cell, as he observed. Whether this property of the cell has functional significance is not yet clear. At any rate, the entire group of the phagocytic mononuclear cells have highly developed surface films by means of which they engulf foreign material. In spite of this close relationship between monocyte and macrophage, both terms must be retained to describe the connective tissues of an animal. This is true because there are relatively young forms, that is monocytes, constantly present in the tissues, corresponding to the monocyte of the blood stream, as well as mature, actively phagocytic cells known as macrophages. The monocytes of the tissues are cells which measure about 20μ in diameter; they have an excentric nucleus and in fixed technique it can be seen that their cytoplasm is basophilic. They have abundant mitochondria and in the living cell there are always some vacuoles that stain in neutral red, indicating a constant function of phagocytosis, though the cell identified as monocyte has never engulfed enough material to be called a macrophage. Monocytes are present in the tissues of the normal animal only in small numbers, often in small foci best seen in the milk spots of the omentum. They are highly capable of multiplication so that their numbers can be readily increased. The more mature phagocytic mononuclear cells, the macrophage or clasmatocyte, are present in the tissues in large numbers. They, and the epithelioid cell, which is a modified form of the monocyte, are considerably larger than the monocyte. The macrophage may be either round or branched, may have much or little phagocytized material. The phagocytized material may be diffusely scattered without pattern in the endoplasm, never in the exoplasm. The lack of pattern is due to the fact that the vacuoles which contain the phagocytized material are in constant streaming motion in the cytoplasm. The phagocytized material may be in particles of uniform or irregular size; it may be in the form of a rosette around the centrosphere. In the typical epithelioid cells, which we have called the third stage (8) of this cell, the phagocytized material is in the form of a rosette of particles so small

that the cytoplasm appears to be uniform in fixed tissues; the nucleus is excentric and there is a marked differentiation between endo- and exoplasm. The epithelioid cell is to be regarded as a pathological type. Every form of the phagocytic mononuclear cell,—the monocyte, the stimulated monocyte, all the different forms of the macrophage, the epithelioid cell, the epithelioid giant cell, and the foreign body giant cell—was induced by the injection of the proteins used in these studies.

Cellular Reactions of Normal Animals to Tuberculo-Proteins in Solution and in Fresh Precipitates

The cellular reactions of normal animals to the tuberculo-protein given in solution or in freshly precipitated state are shown in Table I.

Four rabbits were given three daily injections of 10 mg. of the protein MA-100. This preparation of protein contains some free polysaccharide. The reaction was studied 5 days after the last injection. The omenta showed a slight increase in monocytes. The omenta of four normal rabbits were studied at the same time for direct comparison in relation to an increase in monocytes after the protein.

Two rabbits were given three daily intraperitoneal injections of 20 mg. of freshly precipitated protein TPA of Seibert. This preparation has been freed from polysaccharide except that which is contained in the protein molecule. In accordance with Dr. Seibert's directions this protein was dissolved by allowing it to stand overnight in distilled water and then precipitating it with dilute acetic acid. The precipitate was washed in water and injected in saline at pH 6.25. The omenta showed a moderate stimulation of monocytes of the type of the four dark cells in the lower part of Fig. 1 from rabbit R 3928. This is a photograph of the fresh omentum taken while the cells were living; it was stained with neutral red so that the dark spots in the photograph represent the particles of phagocytized material stained red. This area from a milk spot of the omentum was not one of the most stimulated zones but was chosen because it shows monocytes with rosettes of vacuoles, monocytes with scattered vacuoles, and, in the upper part, a branched macrophage. In the sections of the omentum of this animal there were a few foci of such stimulated monocytes, epithelioid cells, as are seen in Fig. 1.

The protein H-1 of Seibert contained an insoluble residue. Under directions kindly supplied us by Dr. Heidelberger, we separated a more soluble part from this preparation. We stirred some of the material with saline at pH 7.5 and let it stand for 24 hours at room temperature; it was then ground thoroughly in a mortar, made up to pH 8, and allowed to stand for 2 days. The material was then centrifuged and the supernatant was precipitated with dilute acetic acid. This precipitate, after it was washed in distilled water, was taken up in saline and used for injection into four rabbits, as shown in Table I. It induced a moderate

TABLE I

Cellular Reactions in Normal Animals to Intraperitoneal Injections of Tuberculo Proteins in Solution and in Suspension

Animal No.	Materials	Number, amount, and site of injections	Interval after injection	Results
Rabbits			days	
R 4108* 4109 4112 4113	MA-100 in solution	3 daily of 10 mg. in 1 cc. saline	5	Peritoneal exudates (means) PMN 2% — Lymphocytes 35% — Monocytes 49% — Serosal cells 14%
				Omentum had a slight increase in monocytes in the milk spots. One animal had a considerable increase in lymphocytes in the omentum and more lymphocytes than monocytes in the exudate
3928 3929	TPA freshly precipitated	3 daily of 20 mg. in 5 cc. saline at pH 6.25	6 and 7	Peritoneal exudates PMN 1% / — — Lymphocytes 6% / 2% — Monocytes 93% / 65% — Serosal cells — / 33%
				Omentum showed a moderate increase in monocytes, in stimulated monocytes and macrophages (Fig. 1); an occasional small tubercle of epithelioid cells; a few giant cells, both epithelioid and foreign body types
4062 4063 4064 4065	H-1 part soluble at pH 8, then freshly precipitated	3 daily of 7 to 8 mg. in 2 cc. saline	6	Peritoneal exudates (means) PMN 0.2% — Lymphocytes 27% — Monocytes 62% — Serosal cells 10%
				Omentum showed a moderate increase in monocytes and stimulated monocytes

* These are serial numbers for the work of the laboratory covering a term of years.

TABLE I—*Concluded*

Animal No.	Materials	Number, amount, aud site of injections	Interval after injection	Results				
			days	Peritoneal exudates				
Guinea pigs				*Total*	*PMN*	*PME*	*Lympho-cytes*	*Mono-cytes*
R 4867	G in so-	1 of 0.5 mg.		21,550	1.4%	0.6%	39%	59%
4868	lution	3 of 0.5 mg. in 0.5 cc. saline	8	82,800	—	1.0%	14%	85%
				Omentum showed a marked increase in monocytes (Fig. 3); some in small foci. 6 days after the last intraperitoneal injection given an intradermal test with 0.1 mg. MA-100; G in solution and G in suspension. All negative in 24 and 48 hrs. but all showed increase in monocytes, least after MA-100, most after G in suspension				
				Peritoneal exudates				
				Total	*PMN*	*PME*	*Lympho-cytes*	*Mono-cytes*
4865	G in sus-	1 of 0.5 mg.		39,300	1%	0.5%	40%	58%
4866	pension	3 of 0.5 mg. in 0.5 cc. saline	8	41,350	1%	15.0%	37%	47%
				Omentum showed a marked increase in stimulated monocytes and epithelioid cells (Fig. 2); some of the epithelioid cells in small tubercles. Skin tests as above				

stimulation of monocytes, both in the peritoneal exudate and in the omentum, quite different from the complex reactions due to the entire protein H-1 and the insoluble residue (Table II).

The protein G from the bacilli was given to four normal guinea pigs, part in solution and part in suspension. This protein is free from polysaccharide except that which is bound in the protein molecule. The guinea pig was used in this experiment for comparison with the reactions to the same material in tuberculous guinea pigs, to be described later (Table III). The effect of this protein, both in solution and in suspension, was a marked stimulation of monocytes, as is clear from the large total numbers of cells in the peritoneal exudates. None of the other animals whose records are shown in Table I had any such increase in the

TABLE II

Cellular Reactions in Normal Rabbits to Intraperitoneal Injections of the More Insoluble Tuberculo-Proteins

Animal No.	Materials	Number, amount, and site of injections	Interval after injection	Results
			days	
R 3888 4070 4072	H-1 entire	3 daily of 20 mg. in 6 cc. saline	5	Peritoneal exudates (means) PMN 11% Lymphocytes 13% Monocytes 74% Serosol cells 1% In the exudate of R 3888 there was one typical epithelioid cell in 500 cells. There was marked irritation of the serosal cells. Cecum covered with exudate; some adhesions; necrotic nodules surrounded by neutrophiles. Omenta showed small foci of necrosis surrounded by neutrophiles and an increase in monocytes, many of them stimulated. A few epithelioid cells and giant cells
4066	H-1 insoluble residue	3 daily of 7 to 8 mg. in 2 cc. saline	4	Peritoneal exudate PMN 20% Lymphocytes 9% Monocytes 70% (of which 27% were stimulated) Other results as above
3886	K in suspension	3 daily of 10 mg. in 6 cc. saline	4	Peritoneal exudate PMN 6% Lymphocytes 34% Monocytes 60% Omentum showed increase in size of milk spots due to monocytes, many of them stimulated (Figs. 4 and 5). A few small tubercles of epithelioid cells; giant cells, both of the Langhans and of the foreign body types
3887 3888	" "	" "	10 and 11	Peritoneal exudates PMN 6% Lymphocytes 59% Monocytes 35% 2% 7% 91% Omentum showed a marked increase in monocytes, both diffusely scattered and in small tubercles (Fig. 7). Some of the tubercles were surrounded by lymphocytes. Many giant cells of both types, some of the foreign body types with many nuclei (Fig. 6)

TABLE III

Cellular Reactions of Tuberculous Guinea Pigs to Intraperitoneal Injections of the Tuberculo-Protein, G

Animal No.	Interval after inoculation with 0.1 mg. Tbc H-37 subcutaneously	Material	Interval after injection	Results
			days	
R 4809 4811	1 mo.	0.5 mg. in solution in 0.5 cc. saline	7	Peritoneal exudates (means of two groups)
4791 4798	" "	0.5 mg. in suspension in 0.5 cc. saline	7	Omenta not tuberculous; increase in stimulated monocytes and epithelioid cells of types seen in Figs. 8 and 9. Reaction noticeably greater in both animals that received the protein in suspension
4749	1 mo. and 3 days	1 mg. in solution in 1 cc. saline	12	Peritoneal exudate of R 4749
4750	" "	1 mg. in solution		All three died within 12 hours in protein shock
4751 4752	" "	1 mg. in suspension		
4753 4754	1½ mos.	3 daily of 0.5 mg. in solution in 0.5 cc. saline	21	Peritoneal exudates (means of two groups)
4775 4787	" "	3 daily of 0.5 mg. in suspension in 0.5 cc. saline	21	Omenta not tuberculous; markedly stimulated with increase in monocytes, stimulated monocytes, epithelioid cells, and giant cells. Foci of lymphocytes in omentum. Reaction markedly greater after the protein in suspension, in which case the milk spots had become confluent. 6 days after the last intraperitoneal injection, all were tested intradermally with 0.1 mg. MA-100; G in solution and G in suspension. They all gave ++++ reactions and the sections 15 days later showed tubercles (Figs. 10, 11, 12), least after MA-100, most and most complex after G in suspension

Results detail for R 4809/4811:

	Total	PMN	Lymphocytes	Monocytes	Serosal cells
	16,755	1%	45%	47%	6%
	20,200*	2%	50%	48%	—

Results detail for R 4749:

	Total	PMN	Lymphocytes	Monocytes
	6100	13%	57%	30%

Omentum not tuberculous; markedly stimulated with an increase in epithelioid cells and a few epithelioid giant cells

Results detail for 4753/4754 and 4775/4787:

	Total	PMN	Lymphocytes	Monocytes	Serosal cells
	5975	4%	77%	14%	5%
	9675	7%	57%	36%	—

* Total count for R 4798 only.

total number of the cells in the exudates.   In estimating the power of this protein to induce cellular reactions, the small amount used must be taken into consideration.   This amount was given for comparison with the reactions in the tuberculous animals.   The differences between the effect of the material in suspension and in solution are brought out in Figs. 2 and 3, showing reactions in the peritoneal exudates of this group.   Fig. 2 is from R 4865 which had received the material in suspension and Fig. 3 from R 4867 which had received the protein in solution.   The two photographs are at exactly the same magnification and the cells were living so that there had been no shrinkage from fixation.   In Fig. 2, which is representative of the exudates of both the animals which received the protein in suspension, the monocytes are markedly enlarged, the diameters varying from two to three times those of the less stimulated monocytes of Fig. 3. The size of these cells is readily estimated from the two lymphocytes which are included in the group in Fig. 2.   The large cell at the left in Fig. 2 with two nuclei illustrates well the method of formation of the epithelioid or Langhans' giant cell which results from the division of the nucleus without cell division, as was shown by Forkner (9).   The single centrosphere shows clearly that this cell with two nuclei was derived from a single monocyte.   Fig. 3 was taken from a group of about 140 monocytes; such clumps of monocytes are quite characteristic of these exudates.   The monocytes in this figure show a considerable variation in the amount and the arrangement of the phagocytized material.   The omenta of these animals showed the monocytes in foci; after the protein in solution there were clumps of monocytes; after the protein in suspension the clumps contained the enlarged, stimulated monocytes and epithelioid cells and hence could be called tubercles, or tubercle-like structures.   The cells from the peritoneal exudates in these normal animals had drained in large numbers into the retrosternal lymph nodes; this was in marked contrast to the condition in the tuberculous guinea pigs, in which the reaction was more restricted to the peritoneal cavity.

All four normal guinea pigs which received the protein G intraperitoneally were given an intradermal injection of 0.1 mg. of three kinds of protein, MA-100, and G in solution and in suspension.   These tests, which were made 6 days after the last intraperitoneal injection, read as tuberculin tests were all negative in 24 and 48 hours, but sections of the areas showed a diffuse increase in monocytes, least after the MA-100 and most after the G in suspension.

Cellular Reactions in Normal Rabbits to Insoluble Tuberculo-Protein

The cellular reactions to the relatively insoluble protein H-1 from the culture media and to protein K from the bacilli are shown in Table II.

The material H-1 was so highly irritating that a careful study was made to see if there were contaminating organisms but none was found.   The percentage of

neutrophiles was higher in the peritoneal exudates corresponding to the areas of necrosis on the cecum and in the omentum. There was a marked increase in monocytes and there were some epithelioid cells and a few giant cells, both of the epithelioid and the foreign body types.

The protein K seemed entirely insoluble at the neutral point and was given in suspension in saline to three rabbits. It proved to be a remarkable stimulant for every type of phagocytic mononuclear cell, as is shown in Figs. 4 to 7. After this material the monocytes and their derivatives were increased, both scattered in the tissues and in small tubercles. Figs. 4 and 5 are from rabbit R 3886 which received three daily injections of 10 mg. each and was sacrificed 4 days later. The peritoneal exudate was increased in amount and was thick and milky from the contained cells. In Fig. 4 are shown stimulated monocytes from this exudate; this photograph was taken from a clump of about 50 cells, no two of which were exactly alike. Compared with the cells of Fig. 3, from an exudate of an animal which received the protein G in solution, it will be seen that these cells were more stimulated. Many of these monocytes contained refractile bodies, white in the photograph, which represent lipid in the cell. In Fig. 5 is a giant cell from the omentum of the same animal. It is clearly of the epithelioid or Langhans type. A typical foreign body giant cell, with many nuclei, is seen in Fig. 6 from rabbit R 3887. The edge of the cell is marked by arrows. A large particle of the material still undissolved is shown completely surrounded by the monocytes which make up this giant cell. In the cell are many foci of vacuoles which are the rosettes of the component monocytes. The nuclei are clear in the right half of the cell, where there is less phagocytized material. A section of the omentum of this same animal is shown in Fig. 7, which makes quite clear the extent of the increase in monocytes over the normal condition. It shows both types of giant cells, those with peripheral nuclei and those in which the nuclei are scattered throughout the cytoplasm. There are some lymphocytes to be seen in this area. The tissues of these animals showed some tubercles of epithelioid cells, some of which were surrounded by lymphocytes and some were not.

Cellular Reactions of Tuberculous Guinea Pigs to Tuberculo-Protein

The tuberculo-protein G in the two forms, in solution and in suspension, was given to three groups of four tuberculous guinea pigs and the tissues were studied 7, 12, and 21 days later. The data on these experiments are given in Table III. The results are to be compared with the effects of the same protein in the normal guinea pigs shown in the fourth and fifth groups in Table I.

The effect of the protein was greater in the tuberculous than in the normal animal, and there was a greater formation of epithelioid cells and giant cells. Thus

there were both quantitative and qualitative differences. It will be noted in general in Table III that the percentage of lymphocytes in the peritoneal exudates was higher in the tuberculous group and more foci of lymphocytes were found in the omenta. In Fig. 8 is shown a milk spot in a film of omentum from a tuberculous guinea pig, R 4791, 7 days after the injection of 0.5 mg. of protein G in suspension. The cells are stimulated monocytes and epithelioid cells. There has been some desquamation of the surface films. In Fig. 9 the cells of the peritoneal exudate of a tuberculous guinea pig, R 4749, are shown 12 days after the injection of 1 mg. of protein G in solution. The photograph shows a greater stimulation of the monocytes than in corresponding exudates of Figs. 3 and 4 from normal animals.

The skin tests which were made with the last group of the tuberculous guinea pigs gave the most instructive material for the differences in the effect of the proteins (Figs. 10, 11, and 12). The tissues were studied 15 days after the intradermal injections had been made. In Fig. 10, which is from guinea pig R 4753 and is taken from the area that received the MA-100, it is clear that there was one small tubercle of epithelioid cells, a little diffuse reaction and a few epithelioid giant cells in the dermis, and a moderately diffuse reaction in the subcutaneous tissue. Figs. 11 and 12 are both from guinea pig R 4775 and show the reaction in the skin to protein G in solution and in suspension. They include the subcutaneous tissue, marked by the fat and the lower part of the dermis. In Fig. 11 it will be seen that the entire subcutaneous level and the lower part of the dermis were filled with epithelioid cells and a few giant cells, a reaction clearly greater than that shown in Fig. 10. These two sections are representative of the entire reaction to MA-100 and G in solution. The small section in Fig. 12 fails to illustrate adequately the far greater extent and complexity of the reaction to the protein in suspension. The area was chosen to show the greater proportion of giant cells; for a wide area both subcutaneous tissue and the dermis were filled with complex tubercular tissue. In none of the sections were there any foci of lymphocytes.

DISCUSSION

These studies show that tuberculo-proteins injected into the tissues of normal animals stimulate the phagocytic mononuclear cells to functional activity and to multiplication. The immediate effect, that occurring within the first few hours, for which the data are not included in this paper, is to call neutrophiles from the vessels to the tissues, as after any other foreign material. This effect is transitory and after a few days, as shown in all the tables, the neutrophiles

were consistently low in the peritoneal exudates. From that time on, the effect of all the materials employed was an increased activity of the phagocytic mononuclear cells. Within this group of cells there was so much variation, every kind of phagocytic mononuclear cell having been involved, as to indicate the complexity of cellular response to these materials.

In general three forms of protein were used: preparations given in solution, in freshly made precipitates, and in suspensions of quite insoluble material. The proteins given in solution, the MA-100 and the protein G, induced a new formation of monocytes in the normal animal. This is in agreement with our other studies (10, 11). After the freshly precipitated proteins, TPA from the media and G from the bacilli, there was not only an increase in monocytes but a marked stimulation of them to functional activity, with formation of epithelioid cells, some of them in small tubercles. The use of the same protein, type G, in solution and in suspension provided a nice illustration of a greater cellular response to the same material as particulate matter, as shown in Figs. 2 and 3.

The proteins H and K, which we have designated as insoluble in Table II, were certainly in a quite different form from the freshly precipitated TPA and G. Injected into the tissues they set up complex reactions involving every type of phagocytic mononuclear cell, monocytes, stimulated monocytes and macrophages, epithelioid cells scattered diffusely and in tubercles, and both forms of giant cells, the epithelioid types with peripheral nuclei and the foreign body forms, some with large numbers of nuclei (Figs. 5 to 7).

It is interesting to note that in the tuberculous animal the activity of every type of protein is markedly enhanced. Thus the protein MA-100 which, in the normal animal, produces only an increase in normal monocytes, in the tuberculous gives rise to some epithelioid cells in the form of tubercles (Fig. 10). The protein G in solution which produced a very few tubercles in the normal animal, gave rise to extensive tubercles in the tuberculous (Fig. 11), while the protein G, in suspension, brought forth still more complex tubercular tissue in the tuberculous animal, with extensive formation of giant cells. This power of the tuberculous animal to enhance such cellular reactions

to tuberculo-protein is in line with the fact that a tuberculous animal will react to the degraded protein of tuberculin (PPD of Seibert), while an animal highly sensitized to tuberculo-protein will not (11–13). These observations suggest that there are materials in the tissues or the circulation of the tuberculous animal not present in the normal one.

For several years we have been studying the effects of introducing chemical fractions derived from acid-fast bacilli into the tissues (14). To these fractions, polysaccharides, lipids, and proteins, we have found four types of cellular reactions. There is first an exudation of neutrophiles from the blood vessels to the tissues; second, a stimulation of the phagocytic mononuclear cells of the tissues; third, a multiplication of fibroblasts; and fourth, a local increase in lymphocytes.

Concerning the granulocytes of the blood stream, none of the materials which we have injected failed to call neutrophiles from the vessels to the tissues. This was likewise true of the menstrua in which we introduced the fractions, namely, distilled water or saline. Of the fractions from the tubercle bacilli, only the polysaccharides gave this reaction alone, and always in greater amount than the menstrua in which they were injected. The polysaccharides themselves did not seem to have any power to stimulate the new formation of cells, but the neutrophiles which they brought into the tissues were phagocytized and digested by monocytes. This provided an opportunity to observe that the monocyte is physiologically adapted to a rapid digestion of engulfed neutrophiles, for the reaction was complete within 48 hours after injection of the polysaccharides. Under two conditions we have found an increase in eosinophiles in the tissues in our experiments, first, after the cells had brought about a partial digestion of the so called wax fractions of the acid-fast organisms (15), and second, in the dermal reactions to injections of tuberculo-protein in sensitized animals (11). No changes in the basophilic leucocytes have been observed after the use of any of the fractions from the tubercle bacilli.

The phagocytic mononuclear cells were stimulated by all of the lipids and proteins. There were, however, marked differences in the activities of these cells after three fractions, namely, the tuberculo-

phosphatide, the so called waxes, and the proteins. After injection of the phosphatide prepared by Anderson (16) there was a uniform reaction of epithelioid cells and their multinucleated derivative, the Langhans' giant cell. The reaction was in the form of tubercles and closely simulated the type of reaction induced by attenuated tubercle bacilli (17–19). These are the hard tubercles of the infection, as described by Rich and McCordock (20). The epithelioid cells induced by the phosphatide regressed en masse, simulating caseation, or the cells died gradually, one by one, and were cleared away as debris.

The reactions to all of the preparations of the so called waxes, the higher alcohols, and the hydroxy acids prepared by Anderson (21) consisted of a multiplication of monocytes and the fusion of them into foreign body giant cells within which these inert substances were gradually disintegrated. The particles of wax were first surrounded by monocytes and then engulfed by their fused surface films (see Fig. 4 in reference 19).

In contrast to these relatively simple and uniform reactions to the lipids, the proteins induce much more complex reactions because they give rise not to one form of the phagocytic mononuclear cells but to every form. The degree of complexity of these reactions varies with the state of the protein, being simplest after the protein in solution, more complex after the proteins as freshly precipitated, and most complex after the more insoluble forms. The proteins induce the formation of monocytes, of stimulated monocytes, of macrophages, of epithelioid cells singly and in tubercles, of giant cells, both of the Langhans and of the foreign body type.

We have found an increase in fibroblasts as a consistent reaction to a material injected only after leprosin, one of the wax-like fractions isolated by Uyei and Anderson (22) from an acid-fast organism obtained from a case of leprosy. The formation of fibroblasts has occurred in all adhesions and in the repair after necrosis. After intraperitoneal injection of leprosin there was induced the new formation of fibroblasts which, in the thin membrane of the omentum, could be seen to grow exactly as they do in tissue cultures (see Plate 34, Fig. 4, in reference 15).

The new formation of lymphocytes has been irregular and inconstant in our series of studies. In the tubercles set up by the phosphatide there was often, but not always, a capsule of lymphocytes around the newly formed tubercles; as seen in this study on the effects of the proteins, some, but not all, of the animals of a group may show an increase in lymphocytes. There was a greater percentage of lymphocytes in the peritoneal exudates of the tuberculous guinea pigs after the injection of the protein than in the normal animals. We have been unable to find that any of the fractions from the tubercle bacillus so far studied induce consistently an increase in lymphocytes.

SUMMARY

1. Tuberculo-protein in solution induces the formation of monocytes in the normal animal and tubercles of epithelioid cells in that which is tuberculous.

2. Freshly precipitated tuberculo-proteins from the culture media and from the bacilli induce a moderate formation of epithelioid cells in normal animals, and more in the tuberculous.

3. Insoluble forms of tuberculo-protein induce complex tubercular tissue in normal animals. This action is enhanced in the tuberculous.

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. EXPLANATION OF PLATES

PLATE 43

FIG. 1. Film of omentum of rabbit R 3928 which had received three injections of 20 mg. of freshly precipitated protein TPA and was studied 6 days later. The cells were living and the vacuoles containing the phagocytized material were stained with neutral red. It is a part of a milk spot and shows stimulated monocytes and macrophages. × 1000.

FIG. 2. Peritoneal exudate of guinea pig R 4865 which had received one injection of 0.5 mg. of protein G in suspension and was studied 8 days later. The cells were living and the vacuoles containing the phagocytized material were stained with neutral red. It shows stimulated monocytes and an epithelioid cell with two nuclei. × 1100.

FIG. 3. Peritoneal exudate of guinea pig R 4867 which had received one injection of 0.5 mg. of protein G in solution and was studied 8 days later. The cells were living and the vacuoles containing the phagocytized material were stained with neutral red. It shows various forms of monocytes. × 1100.

FIG. 4. Peritoneal exudate of rabbit R 3886 which had received three daily injections of 10 mg. of protein K in suspension and was studied 4 days later. The cells were living and the vacuoles containing phagocytized material were stained with neutral red. It shows various forms of monocytes, more stimulated than those of Fig. 3. × 1000.

FIG. 5. Film of omentum from the same animal as Fig. 4, to show an epithelioid giant cell. The cells were living and the rosette of vacuoles was stained with neutral red. The nuclei are not well shown because the vacuoles are in focus. × 1000.

FIG. 6. Foreign body giant cell in a milk spot of the omentum of rabbit R 3887 which had received three injections of 10 mg. of protein K and was studied 10 days later. The cell was living and both the plaque of the protein and the vacuoles containing the material phagocytized in the giant cell were stained with neutral red. The edges of the giant cell formed by the fusion of many monocytes are indicated by arrows. × 1000.
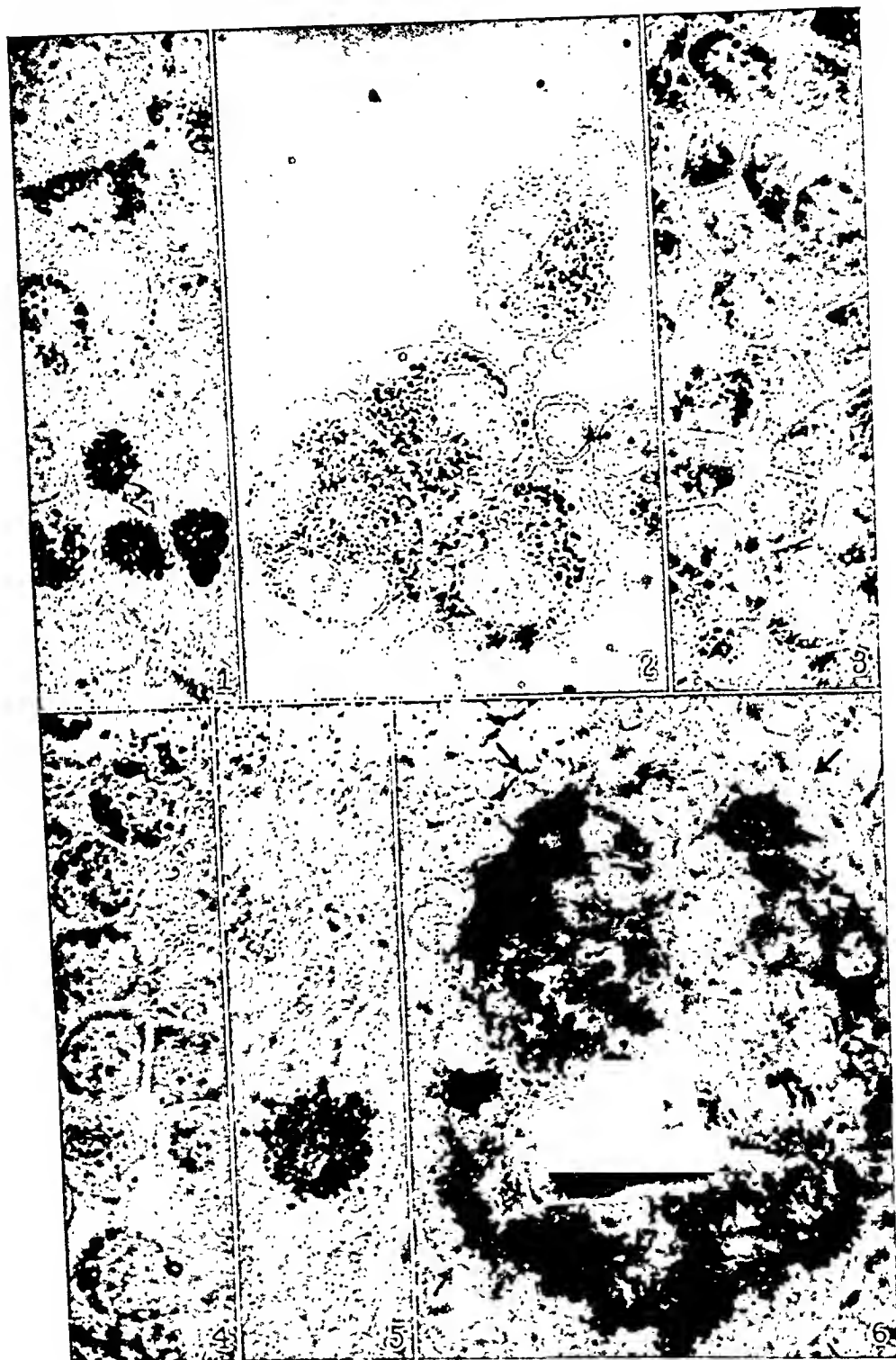
(Sabin: Cellular reactions to tuberculo-proteins)

PLATE 44

FIG. 7. Section of the omentum of the same animal as in Fig. 6. It shows that the omentum was thickened and that there had been a marked increase in monocytes and the formation of giant cells of both types. There were also some lymphocytes. Stained with Foot's modification of Masson. × 325.

FIG. 8. Film of omentum of a tuberculous guinea pig, R 4791, which had received 0.5 mg. of protein G in suspension and was studied 7 days later. The cells were living and the vacuoles which contained the phagocytized material were stained with neutral red. It shows stimulated monocytes and epithelioid cells. There has been a marked desquamation of surface films of the cells. × 1100.

FIG. 9. Peritoneal exudate of a tuberculous guinea pig, R 4749, which had received 1 mg. of protein G in solution and was studied 12 days later. The cells were living and the vacuoles which contained the phagocytized material were stained with neutral red. It shows stimulated monocytes and epithelioid cells. × 1100.

FIG. 10. Section of the skin of a tuberculous guinea pig, R 4753, which had received an intradermal injection of 0.1 mg. of MA-100 15 days before the animal was sacrificed. It shows a tubercle of epithelioid cells and a few giant cells. Stained with Foot's modification of Masson. × 100.

FIG. 11. Section of the skin of a tuberculous guinea pig, R 4775, which had received an intradermal injection of 0.1 mg. of protein G in solution. It shows a marked infiltration of dermis and subcutaneous tissues with epithelioid cells. Stained with Foot's modification of Masson. × 100.

FIG. 12. Section of the skin of the same animal as in Fig. 11, in the area which had received 0.1 mg. of the protein G in suspension. It shows tubercular tissue of epithelioid cells and giant cells. Stained with Foot's modification of Masson. × 100.
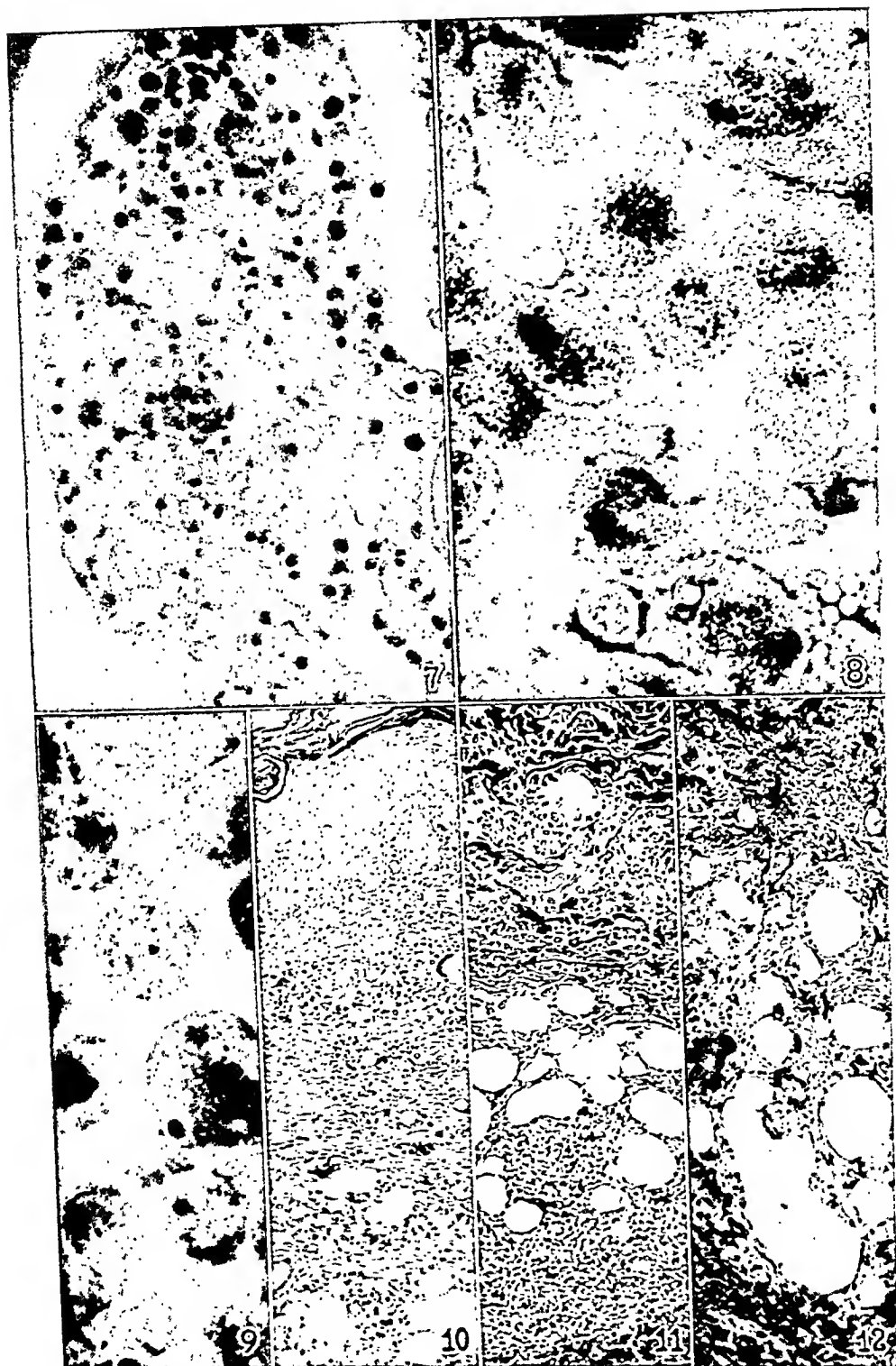
(Sabin: Cellular reactions to tuberculo-proteins)

CELLULAR REACTIONS TO DEFATTED TUBERCLE BACILLI AND THEIR PRODUCTS*

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PLATES 45 AND 46

Since 1926 we have been following the cellular reactions to various fractions from tubercle bacilli. The present study completes our general biological survey of the main lipoidal fractions of acid-fast organisms as analyzed by Dr. Anderson.

In Chart 1 is given in briefest possible outline the chemical procedures used by Dr. Anderson to obtain the materials we have studied. It represents only the end products of his analyses; for example, each of the solvents used originally, namely, alcohol-ether, acetone, and chloroform, extracted some of all the forms of lipids in the bacilli and only predominantly the type indicated on the chart. The separation of phosphatide, acetone-soluble fat, and the so called wax from each other after these primary mixed extractions has been described in Dr. Anderson's publications, of which a complete list to date is appended (1–52). The solvents used are shown on the chart in black face type; the residues and the extracts which are acid-fast are enclosed with double lines, while those which are non-acid-fast are enclosed with single lines.

The cellular reactions to the tuberculo-phosphatide, the acetone-soluble fat, and to the so called waxes have been published previously (53–77). The present study involves the biological reactions to the lipids and polysaccharides from the second residue, the so called defatted bacilli.

853

Materials and Methods

The defatted bacilli lack all the lipids that can be removed by neutral solvents such as alcohol-ether and chloroform, but they are in reality only partially defatted, for they are still strongly acid-fast.   They still contain what Dr. Anderson
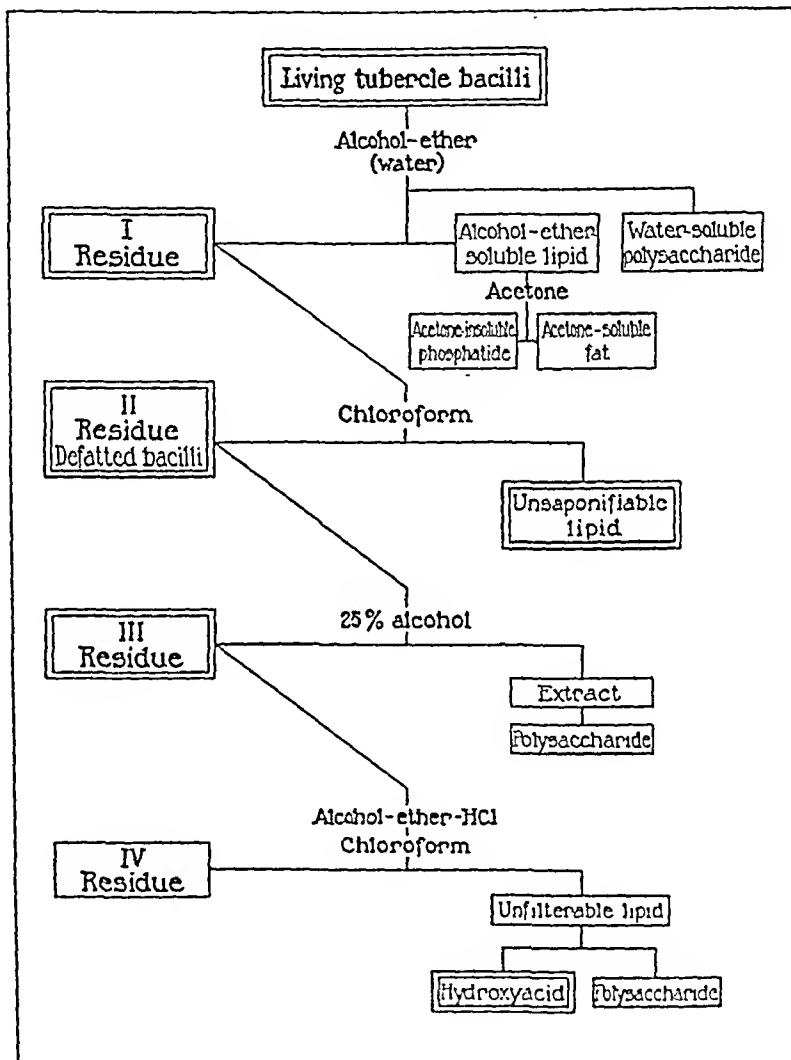
CHART 1

has called the "firmly bound lipid." They were first treated with 25 per cent alcohol by him, which gave an extract which proved to be predominantly of polysaccharide. The residue, the third on the chart, was also of strongly acid-fast organisms. This third residue was then treated with alcohol-ether-HCl after

which it was possible to remove the firmly bound lipid with ether and chloroform; only the chloroform is recorded on the chart. This lipid he found to be unfilterable; it could be split into about equal parts of an hydroxy acid and a polysaccharide. The remaining residue was non-acid-fast.

It has long been known that it is difficult to obtain materials from tubercle bacilli which are free from acid-fast organisms. In the case of the polysaccharides this offers no obstacle because, the material being soluble in water, the organisms can be readily removed by filtration through candles. With the lipids, on the other hand, which must be filtered in lipoidal solvents, one cannot be sure that every filtration will remove the last traces of bacilli, since a lipoidal solvent alters the conditions of filtration. We have described in another paper (74) the methods by which it was possible to free the tuberculo-phosphatide from the last traces of bacilli. Dr. Anderson filtered the material through Chamberland candles but not every preparation was entirely free of organisms even after such treatment. We have found that last traces of the organisms can be removed by centrifugation if the lipid is dissolved in a mixture of solvents the specific gravity of which allows the sedimentation of tubercle bacilli. For this purpose we have used chloroform three parts and ether seven parts. After centrifuging at 3600 R.P.M. for 3 hours in this mixture the bacilli have been found in the sediment and not in the supernatant fluid. As a further check on the presence of a few bacilli in the materials we have stained the tissues of the animals which have received lipids for the acid-fast reaction; when bacilli were present in the materials they were usually to be found in giant cells during the first week after the injections. In the case of the phosphatide, we presented further evidence that the material we used lacked bacilli in any considerable numbers by showing that the phosphatide itself injected intradermally did not sensitize guinea pigs to the tuberculo-protein which is contained in the bacilli, while when minute traces of the protein were added to the phosphatide, sensitization did occur (74).

In the materials described in this report tubercle bacilli were found as follows: The second and third residues were predominantly of acid-fast organisms, markedly beaded and looking much like the bacilli in old cultures. The fourth residue from which the so called firmly bound lipid had been removed by ether-chloroform after the material had been treated with HCl, was predominantly of non-acid-fast material, but there were a few residual organisms which were found both in the material itself and in the tissues. This fourth residue consisted of two kinds of material—first, there was much granular material without any suggestion of bacillary form, which took the green counterstain in the Ziehl-Neelsen technique; and secondly, larger masses of granular material which became a dull purple color in the same technique. These larger masses seemed to have shadows 'of the bacillary form. The acid-fast organisms found were only faintly acid-fast and were described as "suggestive of tubercle bacilli."

The original preparation of the unfilterable lipid was itself not acid-fast but contained a considerable number of acid-fast bacilli; these were separated out

from the material by centrifugation, as described above, until none was found either in the material itself or in the tissues.

The unfilterable lipid and the hydroxy acid obtained from it offer interesting materials for the study of acid fastness, because the unfilterable lipid is non-acid-fast and the hydroxy acid is acid-fast. We had difficulties in applying the acid-fast technique to the chemical fractions to obtain comparable results until we found that preparations made with the standard technique varied markedly in accordance with the melting point of the materials tested. Specifically, those materials which are highly acid-fast, the unsaponifiable wax and the hydroxy acid of these experiments, have so low a melting point, 54–56°, that the heating involved in the standard technique melts the material and makes it roll into droplets or spheres much too dense to analyze any structure in them. The method we have found best is to obtain a dilute solution of the material in its appropriate solvent, which is chloroform for the so called waxes, and spread this solution evenly on a clean slide. The slide is then thoroughly dried in an incubator at 37° and then covered with fuchsin and allowed to stand at 37° for from 12 to 16 hours. The decoloring, washing, and counterstaining are done by letting the fluids flow gently onto the end of the slide and run over the surface of the films. By this method it can be seen that the unfilterable lipid spreads in an exceedingly thin homogeneous film on the slide and that it not only does not retain the fuchsin but it reacts readily to the counterstain. The hydroxy acid, on the other hand, retains the fuchsin and rejects the counterstain. Under oil immersion lenses it could be seen that the hydroxy acid was made up of finely but slightly irregularly granular material which is acid-fast; it contained a very few clumps of acid-fast bacilli.

RESULTS

Defatted Bacilli and Third Residue.—The cellular reactions to the defatted bacilli and to the next residue in series were so similar that we have included the records of both substances in Table I. The bacilli were from the human strain, A-14. The only difference between the two materials was that the reactions to the third residue, while exactly the same in kind, were consistently a little greater in amount. There were more cells, and more bacilli were found in the giant cells. The defatted residue was in the form of a white granular material moderately clumped; the third residue was slightly yellowish and was in larger clumps. Both were from human tubercle bacilli, strain A-14. Both were rubbed into suspensions, which settled so quickly that it was better to weigh the material for each injection separately; some of the suspension was unavoidably lost by sticking to

TABLE I

Cellular Reactions to Defatted Tubercle Bacilli (Human Strain, A-14) and to the Third Residue

Rabbit No.	Material	Number, amount, and site of injections	Time Interval after intraperitoneal injection	Results
			days	
R 5407[*] 5408	Defatted tubercle bacilli	1 of 1 mg. in 1 cc. saline i.p. and 6 of 0.1 mg. in 0.1 cc. saline i.d.	10	The last intradermal injection was like a + or ++ tuberculin test. The dermal lesions showed neutrophiles, monocytes, and giant cells, the latter containing acid-fast bacilli. The peritoneal exudates were of monocytes and lymphocytes in about equal numbers. The omenta showed foci of monocytes and epithelioid cells surrounded by lymphocytes, and some giant cells
5409 5410	Third residue			
5542 5543	Defatted tubercle bacilli	6 of 1 mg. in 1 cc. saline i.p.	12	4 days after the last injection animals tested with 1 mg. of MA-100 i.d. and gave +++ and ++++ reactions. The peritoneal exudates were of monocytes and lymphocytes in about equal numbers. The omenta showed abscesses; tubercles of epithelioid cells with infiltration with lymphocytes; giant cells containing acid-fast bacilli
5540 5541	Third residue			

Peritoneal exudates of the two groups showed:

Rabbit No.	Material	Number, amount, and site of injections	Time Interval		PMN	Lymphocytes	Monocytes
6044 6045	Defatted tubercle bacilli	1 of 20 mg. in 5 cc. dist. H$_2$O i.p.	8		43%	4%	52%
6046 6047	Third residue				35%	2%	62%

About 10% of the monocytes had phagocytized neutrophiles. The omenta and body walls had massive and complex reactions; many abscesses, monocytes, and epithelioid cells both scattered and in tubercles; free and phagocytized neutrophiles; edema; giant cells with acid-fast bacilli

i.p. = intraperitoneal; i.d. = intradermal.

[*] These are serial numbers covering the work of the laboratory for a term of years.

the wall of the syringe. There were three experiments with approximate dosages and intervals, as shown in Table I. The material had power to sensitize to tuberculo-protein, shown both in the increasing size of the reactions to intradermal injections of the material itself in the first experiment, and in the reactions to tuberculo-protein MA-100 in the second experiment. The cellular reactions were exceedingly complex and are shown in Figs. 1, 2, 5, and 6. There were small abscesses of neutrophilic[1] leucocytes; more often there were tubercles of epithelioid cells and giant cells infiltrated with neutrophiles, as in Fig. 1. This tubercle was very small; others measured 2 to 3 mm. in diameter. Some of the tubercles showed new fibers; other areas showed a diffuse infiltration with monocytes, as in Fig. 2. There were many giant cells, some in the tubercles, as in Fig. 1, some quite free in the tissues. One of these giant cells is shown in Fig. 5; the stain was hematoxylin and eosin and the deeply eosinophilic cytoplasm indicated that the cell was badly damaged or dead. This cell was so large that it appeared in several serial sections, and so a part of it is also shown in Fig. 6, stained for tubercle bacilli. It contained only a few bacilli as compared with many of the giant cells, for in some of them there were such dense masses that no photograph could resolve them into separate organisms.

The Products of Extraction with 25 Per Cent Alcohol.—The extract obtained by treating the defatted bacilli with 25 per cent alcohol was a brown, salve-like material from which Dr. Anderson obtained a polysaccharide. The cellular reactions to the original extract and to the purified material obtained from it are shown in Table II.

The reactions in these two experiments were typical of the effect of polysaccharides. The tissues were studied 24 hours after the injections were made. There was a high percentage of neutrophiles in the peritoneal exudates and many of these extravasated neutrophiles had been engulfed by the monocytes in the milk spots of the omentum.

Fourth Residue and Its Derivatives.—In Table III are shown the cellular reactions to the fourth residue and to the unfilterable lipid and its two derivatives, namely, the hydroxy acid and the polysac-

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[1] The term neutrophile is used for the pseudoeosinophilic leucocyte of the rabbit.

charide.   These materials were from the human strain, A-10.   The fourth residue was a fine, granular material which was non-acid-fast but contained a few tubercle bacilli.   The four rabbits which received the fourth residue showed a moderate increase in monocytes in the milk spots of the omentum, and some infiltration of these milk spots with neutrophiles.   The number of the neutrophiles varied in the different animals.   There were some giant cells containing acid-fast bacilli.

TABLE II

Cellular Reactions to a 25 Per Cent Alcoholic Extract and a Polysaccharide

Rabbit No.	Material	Number, amount, and site of injections	Time Interval after intraperitoneal injection	Results
			hrs.	
R 5704 5705	25% alcoholic extract from human tubercle bacilli, strain A-14	20 mg. in 1 cc. saline i.p.	24	Increase in neutrophiles in the peritoneal exudates, averaging 82%; monocytes, 15%; lymphocytes, 1%; serosal cells, 2%. Marked phagocytosis of neutrophiles in milk spots of omentum
5664 5665 6118	Polysaccharides from human tubercle bacilli, strain A-10	10 mg. in 1 cc. saline i.p.	24	Increase in neutrophiles in peritoneal exudates. Neutrophiles, 58%; lymphocytes, 3%; monocytes, 39%, of which 10% had phagocytized neutrophiles. Marked phagocytosis of neutrophiles in milk spots of omentum
6111 6118 6049		10 mg. in 5 cc. saline i.p.		

The unfilterable lipid gave a moderate reaction consisting of an increase in monocytes and some giant cells.   Practically every milk spot contained a giant cell, as seen in films of the omentum.   As shown in Fig. 4, some of the giant cells were surrounded by neutrophilic leucocytes.   This is interesting because this lipid is made up of two substances, an hydroxy acid which itself gives rise to giant cells, and a polysaccharide which attracts neutrophiles from the

TABLE III

Cellular Reactions to the Fourth Residue, the Unfilterable Lipid and Its Derivatives, the Hydroxy Acid, and the Polysaccharide, Human Strain A-10

Rabbit No.	Material	Number, amount, and site of injections	Time interval after intraperitoneal injection	Results
R 5706 5707	Bacterial residue IV (contains a few acid-fast tubercle bacilli)	20 mg. in 1 cc. saline i.p.	*days*	Moderate increase in monocytes and some infiltration of tissues with neutrophiles; a few giant cells containing acid-fast bacilli
6335 6336		20 mg. in 5 cc. dist. H₂O i.p.	7	
5676 5677	Unfilterable lipid	20 mg. dry powder through operative incision under anesthesia, i.p.	7	Moderate reaction; peritoneal exudates contain neutrophiles, 33%; lymphocytes, 3%; monocytes, 63%. Omentum, diffuse increase in monocytes; some giant cells surrounded by neutrophiles
5702 5703	Hydroxy acid (contains very few acid-fast tubercle bacilli)	" "	7	Moderate reaction; peritoneal exudates contain neutrophiles, 3%; lymphocytes, 4%; monocytes, 92%. Omentum showed diffuse increase in monocytes; some giant cells surrounded by eosinophiles
6029 6030 6115 6116	Polysaccharide from unfilterable lipid	10 mg. in 5 cc. saline i.p.	24	The peritoneal exudates of the two groups differed in proportion of free neutrophiles: PMN 80%, Lymphocytes 1%, Monocytes 18%
125 26		10 mg. in 0.5 cc. dist. H₂O i.p.		PMN 24%, Lymphocytes 6%, Monocytes 68%. The omenta showed neutrophilic leucocytes and their phagocytosis by monocytes

vessels. The reactions shown in Fig. 4 suggest that at this interval, 7 days after the injection, the giant cells had been able to break down the lipid enough to liberate some polysaccharide.

The cellular reaction to the hydroxy acid is shown in Fig. 3. It consisted of the formation of foreign body giant cells, some with a few nuclei, some with many. There were a very few bacilli in this material but in our opinion there were far more giant cells than could be caused by them. No bacilli were found in the tissues. The foreign body giant cell is the characteristic reaction to the waxes from tubercle bacilli. At a certain stage the tissues around these giant cells become infiltrated, not with neutrophiles but rather with eosinophiles which are marked by arrows in Fig. 3. The polysaccharide from the unfilterable lipid gave rise to the same reactions as were described above for the polysaccharide from the defatted bacilli.

DISCUSSION

The defatted tubercle bacilli were of course rich in protein since this is the material from which the proteins of the organisms are obtained. No studies were made of the proteins from this material, however, since we have studied the cellular reactions to the bacillary proteins from material given to us by Dr. Michael Heidelberger (76).

The defatted tubercle bacilli were only partially defatted for they still contained enough lipid to be acid-fast. The defatted tubercle bacilli and the third residue gave cellular reactions which were much like those known to characterize the reaction to heat-killed tubercle bacilli. They were complex and consisted of abscesses; tubercles of monocytes and epithelioid cells; of monocytes diffusely scattered in the tissues; of giant cells, many of which contained masses of the tubercle bacilli. Some of the tubercles were infiltrated with neutrophiles. Most of the acid-fast organisms were to be found in the giant cells; occasionally one or two were found in a monocyte, and more rarely still, a few were free. The last residue, from which the firmly bound lipid had been removed, was relatively inert material. It was clear that the protein in this residue must have been much altered from its original state; this residue, however, still retained the property of stimulating a new formation of monocytes.

The unfilterable lipid and hydroxy acid gave interesting material for the study of cellular reactions because the unfilterable lipid was made up of the hydroxy acid plus a polysaccharide.   The basic cellular reaction to the hydroxy acid was the formation of foreign body giant cells in greater numbers than could be induced by the small content of bacilli.   The unfilterable lipid gave such giant cells, and after a week's time the tissues became infiltrated with neutrophiles, that is, reactions to this lipid included those of its constituents, the hydroxy acid and the polysaccharide.

SUMMARY

The cellular reactions to defatted tubercle bacilli are complex and like those to heat-killed whole tubercle bacilli.

The firmly bound lipid, when removed from these organisms, is non-acid-fast; it contains an hydroxy acid which is acid-fast and a polysaccharide, which is not.

This hydroxy acid gives rise to foreign body giant cells and the tissues eventually become infiltrated with eosinophiles.

The polysaccharides, both from the defatted bacilli and from the unfilterable lipid, call neutrophiles from the blood stream.

The reactions to the unfilterable lipid include those of both its constituents.

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EXPLANATION OF PLATES

PLATE 45

FIG. 1. Section of the omentum of a rabbit, R 6046, which had received 20 mg. of the third residue, strain A-14, in 5 cc. distilled water, intraperitoneally. The tissues were studied 8 days later. It shows one of the smallest tubercles; it has a giant cell; it consists of epithelioids infiltrated by neutrophiles. It has a sharp border of fibers. Foot's modification of Masson stain.   × 1000.

FIG. 2. Section of the omentum of a rabbit, R 6047, which had received the same material as in Fig. 1. The tissues were studied 8 days later. It shows an area with a diffuse infiltration of monocytes. Foot's modification of Masson stain.   × 1000.

FIG. 3. Section of the omentum of a rabbit, R 5702, which had received 20 mg. of the hydroxy acid, intraperitoneally. It was introduced as a dry powder through an incision under anesthesia. The tissues were studied 7 days later. It shows a foreign body giant cell, some monocytes, and an infiltration of the tissues with eosinophiles, which are marked by arrows. Foot's modification of the Masson stain.   × 1000.

FIG. 4. Section of the omentum of a rabbit, R 5677, which had received 20 mg. of the unfilterable lipid, intraperitoneally. It was introduced as a dry powder through an incision, under anesthesia. The material had had the bacteria removed by centrifugation. The tissues were studied 7 days later. It shows a foreign body giant cell and a marked infiltration of the tissues with neutrophiles. Stained with Giemsa.   × 1000.

FIG. 5. Section of a nodule on the intestine of a rabbit, R 5410, which had received 1 mg. of the third residue, intraperitoneally. The tissues were studied 10 days after the injection. It shows a dead giant cell in the center of a tubercle of epithelioid cells. This tubercle contains a few neutrophiles. Stained with hematoxylin and eosin. × 1000.

FIG. 6. Section of a part of the same giant cell as shown in Fig. 5, stained to show the acid-fast bacilli within it. The technique used was that described by Fuller (77). × 1000.
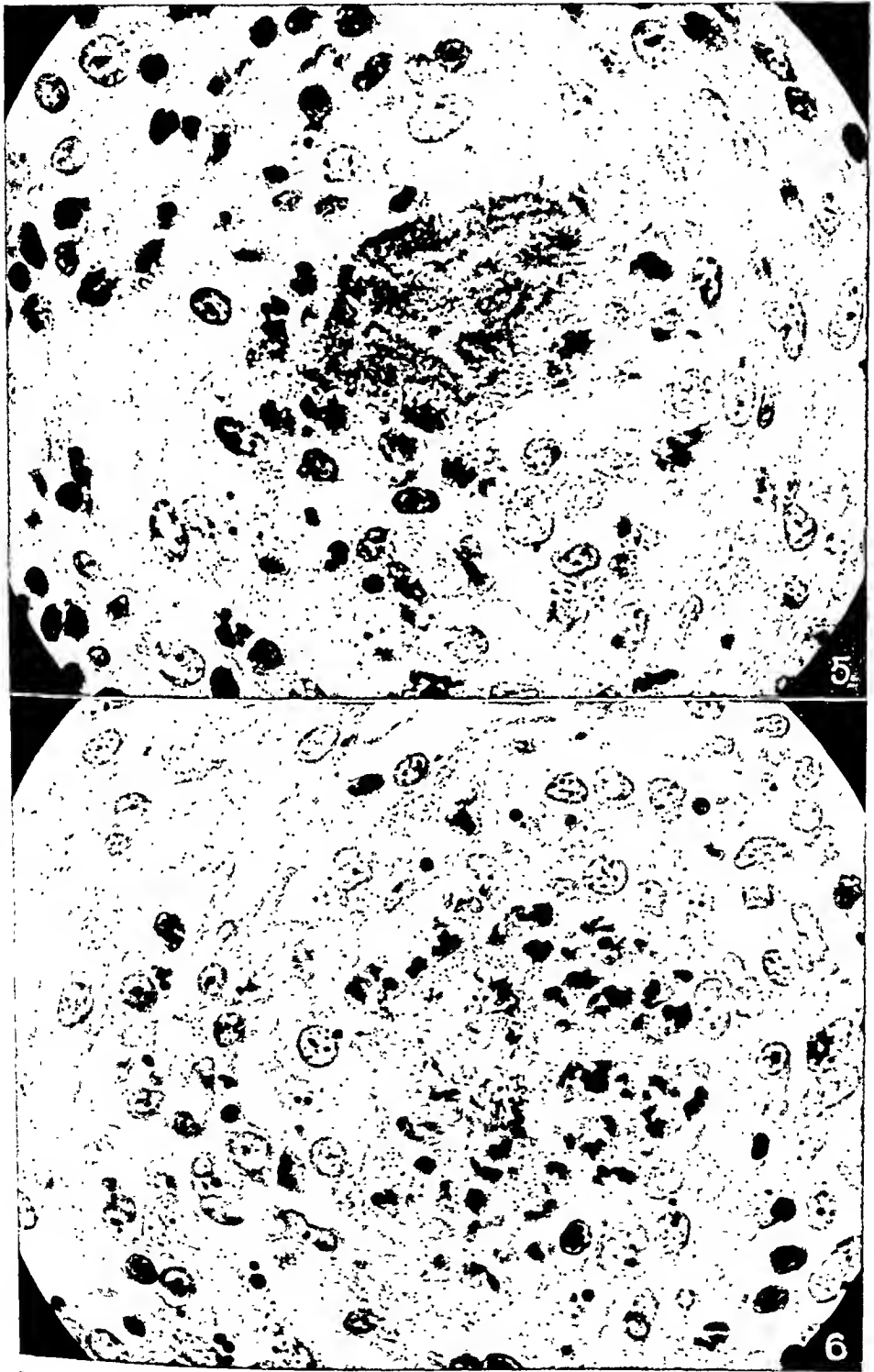
(Sabin and Joyner: Cellular reactions to defatted tubercle bacilli)

NORMAL AND PATHOLOGICAL FACTORS INFLUENCING THE SPREAD OF A VITAL DYE IN THE CONNECTIVE TISSUE

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The pulsation of blood vessels increases the spread of vital dye through a tissue (1). Further, in promoting interstitial dye spread, the mechanical action of the pulse seems to be more important than the pressure of the blood or its rate of flow. The pulsation of blood vessels also favors the movement of fluid through tissues, as indicated by increased formation and flow of lymph (2).

The mechanism of the interstitial movement of fluid or other substances is not understood. It has seemed reasonable to assume that a study of the variations in the rate or character of the spread of vital dyes through tissues, under differing conditions which change the total fluid content of the body, may give evidence bearing upon the problem.

In the present work we have studied in living tissues, under varying physiological and pathological conditions, the spread of a vital dye, pontamine sky blue,[1] introduced without pressure, and in solution isotonic with blood. This dye is the most indiffusible one we have found and it has been employed by us in previous work on the permeability of the lymphatics and on lymph flow (1–8). The dye does not stain the formed elements of the tissues during the short time of the experiments.

Methods

The purification and preparation of the dye solution is simple. The crude dye, obtained in solid form as a sodium salt with various impurities, is dialyzed for 2 or 3 days through parchment, against hot running water. From time to time the

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[1] Du Pont Chemical Company.

flow of water is shut off and after a few minutes samples taken close to the membrane are tested for chlorides. If chlorides are present the dialysis is continued against hot tap water until the test becomes negative. For 2 more days further dialysis is done against many changes of distilled water. This is carried out in the ice box to inhibit bacterial growth.

As shown by freezing point determinations, a 21.6 per cent aqueous solution of this dye was isotonic with blood. A 2 per cent solution, also isotonic with blood, was prepared by suitably diluting the strong dye mixture with Tyrode's or Locke's solution.

In earlier work (1), a method was devised by which we could bring test fluids into the superficial connective tissue without the exertion of pressure. This method, improved and modified, has been employed for the present studies. Mice of about 25 gm. body weight were anesthetized with sodium luminal, injected intraperitoneally, in 2 per cent solution, 0.125 cc. for each 10 gm. of body weight. They were then placed upon plasticine moulds so made that the animal lay with its ears spread horizontally on white porcelain plaques under the binocular microscope (9, 3). With exceedingly fine dissecting needles minute punctures were made through the epidermis into the subpapillary layer of the corium, opening a direct pathway to the tissue, and under a binocular microscope minute amounts of the isotonic 2 per cent pontamine sky blue solution in Tyrode were instilled into the puncture wounds from a micro pipette. To introduce it into the tissues the tip of the pipette was brought into gentle contact with the stab wound allowing the colored solution to drain by capillarity into the wound until it was just filled. Under these circumstances the dye promptly appeared as a superficial spot of color situated just beneath the epidermis, lying in the tissues under no pressure other than that incidental to osmosis and diffusion. As will be seen below, when first observed these spots varied from 0.8 to 1.2 mm. in diameter and were approximately 3 times larger than the puncture wounds which were 0.3 to 0.4 mm. in diameter. The dye lay in a shallow layer with sharply defined, smooth margins, which gradually paled, as the dye spread through the tissues, although the margins remained well defined for over an hour. Gradually the whole spot of dye became paler throughout. For these reasons experiments were carried on only while the margins of color were well defined. These minute pools of color will be referred to as dye spots or maculae. In size and shape they have much general resemblance to the common pigment maculae of skin.

Standard Micro Maculae.—In our first experiments scores of dye maculae were placed in the ears of mice, but one in each ear, and their spread measured as will be detailed below. We tried to make them the same size by simply holding the micro pipette against the tissue, until enough dye had entered by capillarity to yield a spot of the desired size, as observed through the binocular microscope. Despite all efforts, maculae of differing sizes developed. Later work showed that the smaller maculae spread more, in relation to their original size, than did the larger ones. Means were sought to form maculae of the same size, containing equal quantities

of dye solution. A micro pipette, of about 0.1 mm. external diameter and 0.07 mm. internal diameter, was prepared, with an even bore near the tip. In the ears of anesthetized mice many micropuncture wounds were made under the microscope, in the usual way, and filled with dye from the pipette until the colored fluid was flush with the surface. To form the average dye spot, 0.8 to 1.2 mm. in diameter in a puncture 0.3 to 0.4 mm. in diameter, the upper meniscus of the column of dye solution in the pipette, 0.07 mm. in diameter, moved on the average 6.3 mm. toward the tip, requiring therefore a volume of 0.024 c.mm. When the subsequent spread of these dye spots was determined it was found that those measuring from 0.8 to 1.2 mm. in diameter spread with great regularity. Thereafter to form maculae of dye we endeavored to use for each spot the same amount of dye solution as indicated above, and except where specially mentioned we subsequently measured only those which fell within the limits of size that are also indicated above. Dye spots made in this way will be called standard micro maculae. Subsequently many similar micro pipettes were made and calibrated so that standard micro maculae could be used for all experiments.

Measuring the Spread of Dye.—Any method of measuring the interstitial spread of dye is open to certain objections. For example, in the ears of mice dye did not spread uniformly in all directions. Movement was greatest toward the base of the ear; it was less in both directions across the ear and least toward the tip. For this reason, in every instance we measured two diameters of each spot, the first in the direction of the longitudinal axis of the ear, that is to say from tip to base,—the other transversely in a direction at right angles to this. At intervals of 5, 20, 40, and 60 minutes after forming the dye spots, these measurements, which will be called longitudinal and transverse diameters of the maculae, were taken through an eyepiece containing an ocular micrometer scale. At the magnifications employed, the spread of the colored margin of a dye spot over one of the smallest linear divisions of the scale, which will be called micrometer units, represented an actual spread of 0.33 mm. in the observed ear.

Within 30 seconds to 1 minute after the introduction of the dye and 5, 20, 40, and 60 minutes later, the outlines of the maculae were drawn with a camera lucida. The areas of the drawings were later determined with a planimeter.

We have indicated the spread of dye in several ways. Two series of charts have been drawn, the first showing in micrometer units the sums of the measurements of the diameters of the spots, the second, in planimeter units, the areas of the spots. The spread of standard micro maculae is shown by continuous lines. As will be seen below, the slope of the curves in both series of charts indicates the rate of dye spread under the varying physiological conditions studied.

In the previous work (1) the spread of dye, observed in the ears of rabbits perfused with pulsatile and non-pulsatile streams of blood, was indicated by dividing the final measurements of the areas of the spots by their initial measurements. One could thus say how many times the dye spot had increased its initial size. In the present work, for comparison, we have calculated the spread of dye in this

way too, using not only the measurements of area but also the sums of the diameters of the spots.

We have further calculated, from the measurements described above, the actual extension of dye through the tissues under differing physiological conditions, to be described below. An example will show best how this has been done. In Text-fig. 1 a, a small oval has been drawn to represent a greatly magnified dye spot, with transverse and longitudinal diameters of 2 and 3 micrometer units respectively, their sum equalling 5 units. In the diagram each micrometer unit, as defined above, is represented by 2 mm., the long or longitudinal diameter of the spot in the longitudinal axis of the ear, the short one in the transverse axis. In Text-fig. 1 b, the spot as it appeared originally is drawn within the large oval

2 Transverse diam.
3 Longitudinal „

a

Transverse diam. now 3 +3 +2 (the original transverse measurement) = 8

Longitudinal diam. now 2 +7 +3 (the original longitudinal measurement) = 12

b

Total 20
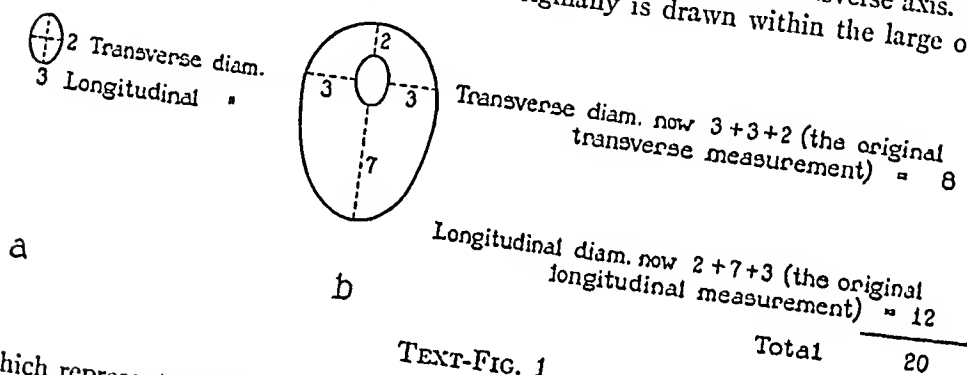
TEXT-FIG. 1

which represents the dye spot after spreading for an hour. The total transverse diameter of the spot, which of course includes the original transverse measurement, has now become 8 units in length, adding to the original 2 units two extensions of 3 units each. The total longitudinal diameter has become 12 units in length, adding to the original 3 units, an extension 2 units long toward the tip of the ear and one 7 units long toward the base. The sum of the diameters has become 20 units. The extension of dye through the tissues, toward the four cardinal points, after the spot was first measured is represented by the dotted lines; 3 units to the right, 3 to the left, 2 upwards, 7 downwards. The sum of these (15 units) expresses better than the sum of the diameters of the entire spot (20 units) the actual spread of dye through the tissues, for it is not dependent upon the initial size of the spot. Calculations of dye spread made in this way will be termed radial spread. Radial spread can be determined by subtracting the initial sum of the diameters from the final diameter sum. Lastly, we have calculated the area of the region of tissue invaded by the color of a spreading dye spot after its formation, by subtracting the original area from the final area, as for example in Text-fig. 1, by subtracting the area of the small oval from that of the large one. The spread of dye expressed in this manner will be called areal spread. We have used this term to express the additional fact that the dye spots are rarely perfectly circular in form to begin with and that they do not, as a rule, spread equally in all directions. Were this so the area of spread could be called annular.

TABLE I

The Spread of Dye through the Connective Tissue of the Mouse Ear in 1 Hour

(2 per cent solution of pontamine sky blue)

(1)	For individual variations see curves in charts (2)	Initial measurement of spots				Ratio of: Final measurement of spots / Initial measurement of spots		Actual increase in size			
		Sums of diameters		Areas		Final diameter sums / Initial diameter sums (7)	Final areas / Initial areas (8)	Radial spread Final sum of diameters minus initial sum of diameters		Areal spread Final areas minus initial areas	
		Micrometer units (3)	mm. (4)	Planimetric units (5)	sq. mm. (6)			Micrometer units (9)	mm. (10)	Planimetric units (11)	sq. mm. (12)
Normal luminal	2a, 2b	5.8	1.7	91	8.1	2.7	3.9	9.7	2.9	267	23.5
Normal ether (hyperemia)	3a, 3b	6.3	1.9	111	9.2	3.1	4.9	13.2	4.0	434	38.2
Normal, actively moving	4a, 4b	6.9	2.1	99	8.7	3.6	8.5	17.4	5.3	739	65.0
Bled luminal	5a, 5b	7.1	2.2	115	10.1	2.0	2.9	6.8	2.1	218	19.2
Dead	6a, 6b	6.0	1.8	92	8.1	1.8	1.8	5.1	1.5	77	6.7
Forming edema	7a, 7b	6.2	1.9	88	7.8	3.6	5.6	16.0	4.9	404	35.8
Bled edema	8a, 8b	6.2	1.9	130	11.4	2.7	3.4	12.7	3.9	316	27.8
Edema already formed	9	6.3	1.9	98	8.6	2.7	4.0	10.8	3.3	297	26.1
Dead edematous	10	6.1	1.9	118	10.3	2.9	3.3	11.5	3.5	270	23.8

Averages of the measurements of the spread of dye spots in each group of experimental animals. The data given in the table are fully described in the text. The figures in column 7 are derived by adding the figures in column 3 to those in column 9 and dividing by the former. The figures in column 8 are derived by adding the figures in column 5 and 11 and dividing by the former.

As will be described below, several groups of experiments were done under physiological conditions differing for each group. We have averaged our calculations from each group of experiments and have expressed in Table I the spread of the dye spots in all the ways mentioned above. This has been done to show that each method of calculation brings out roughly the significant differences in the spread of dye spots under the differing physiological conditions. The table should be used in conjunction with the descriptions of the experiments to be reported below. In the table columns 3 and 4 give the averages of the initial measurements of the sum of the diameters of the spots in micrometer units and millimeters. The averages of the initial areas, in planimeter units and square millimeters, are given in columns 5 and 6. In columns 7 and 8 the average increase in the size of the dye spots in each group of animals is expressed by dividing the final measurements by the initial ones. For example, the figures in the first line in columns 7 and 8 show that the spots of dye in normal animals, under luminal anesthesia, were 2.7 times larger at the end of the hour than at the beginning, as expressed in terms of the increase in diameter sums, and 3.9 times larger in area. In columns 9 and 10, the averages of the radial spread of dye spots in the ears of mice subjected to the various experimental procedures is shown both in micrometer units and in millimeters, and in columns 11 and 12 the averages of areal spread of the maculae in planimeter units and in square millimeters. It has just been noted that all the methods to calculate dye spread show a rough agreement. From this we feel that the method employed in our previous work (1) adequately expressed the differences in dye spread that were observed there.

In a few experiments spots of dye were also made with the aid of uncalibrated pipettes and their spread measured in the usual way. As already mentioned, they varied much in size. The initial measurements of some of the maculae made with uncalibrated pipettes matched those of the standard dye spots, and when this occurred the former spread at the same rate as the latter. Under similar conditions, as will be seen below, the differences in the rate of spread of maculae of equal original size, whether standard spots or those formed with uncalibrated pipettes, were never as great as those caused by alterations in the experimental conditions. As result the spread of both kinds of maculae has been plotted on some of the charts, the standard spots by continuous lines, the others by dotted lines. These charts indicate that the spread of maculae of the same size is similar.

The dotted lines have been included in the charts of this report to show further that, in the preceding work, we were justified in comparing the spread of spots of similar size but made with uncalibrated pipettes.

In earlier papers we have noted that intradermal injections of dye, when made with a hypodermic needle, are to a large extent intralymphatic (6, 7). Superficial lymphatic capillaries are torn or ruptured by the needle and dye enters them directly. In the present work, using a micro dissecting needle and micro pipettes, the puncture wound in the skin could be made in the ear of the mouse about three times out of five without rupturing a superficial lymphatic capillary. All instances were discarded in which dye placed in the puncture wound appeared in a lymphatic.

In other experiments, detailed below, in which edema was produced, droplets of fluid frequently escaped from the tissues through the puncture wound, carrying with them the dye solution. These instances too were discarded.

A single spot of dye was placed in the connective tissue of each ear and in the same relative location in all the experiments. This was done in order to keep the results comparable in every possible way, for we had observed that dye injected into the skin of various portions of the body spread with unequal rapidity. The rate was especially fast where the skin was subjected to the respiratory movement. The ears of mice were ideal to work with, for they could be kept completely at rest, while furthermore their tissues and even the finest blood capillaries were readily observable in light from a carbon arc filtered and cooled by passage through 4 cm. of Mohr's solution and reflected onto the ears by a concave mirror. The organs were constantly watched for evidence of circulatory changes and for edema. Varying degrees of reflex hyperemia followed upon the making of the puncture wounds. Under the binocular microscope, areas distant from the maculae were gently prodded from time to time with a micro needle for evidence of pitting in the skin, and at the end of the experiment this was done to the tissue immediately about them. The onset of edema could readily be seen. All findings from ears showing signs of it will be considered separately, as will those from hyperemic ears.

The Spread of Dye through Tissues as Affected by Changes in Their Physiological State

The spread of 2 per cent solutions of pontamine sky blue was tested in the ears of normal mice and in those of animals subjected to various procedures affecting the total body water content.

A 21.6 per cent aqueous solution, isotonic with blood, was employed, diluted to 2.0 per cent with Tyrode's solution. The spread was first measured in mice of about 25 gm. anesthetized by the intraperitoneal injection of 0.25 cc. of a 2 per cent solution of sodium luminal. In Text-fig. 2 a the spread of 9 standard micro maculae in the ears of the luminalized mice have been plotted in continuous lines representing the spread as shown by the sum of the diameters for each 20 minutes of an hour. The chart shows further the spread of 5 maculae which happened to be of about the same size, made under similar conditions with uncalibrated pipettes. The average of the initial measurements of the sums of the diameters of these maculae was 5.8 units and at the end of the hour 15.8 units. The size of the spots, as expressed by these measurements, had increased 2.7 times.

In Text-fig. 2 b the continuous lines, Nos. 1 to 9, represent the spread of the 9 quantitative maculae designated by the same numbers in Text-fig. 2 a, but plotted in terms of planimetric units determined as already described. The curves represent increase in area. The average of the area determinations at the end of the hour was 3.9 times that of the average of the initial measurements.

The averages of the actual movement of dye through the tissues, in these and subsequent experiments (radial spread and areal spread), are presented in Table I, columns 9 to 12. The radial spread averaged 9.7 units, or about 2.9 mm., in the experiments just described.
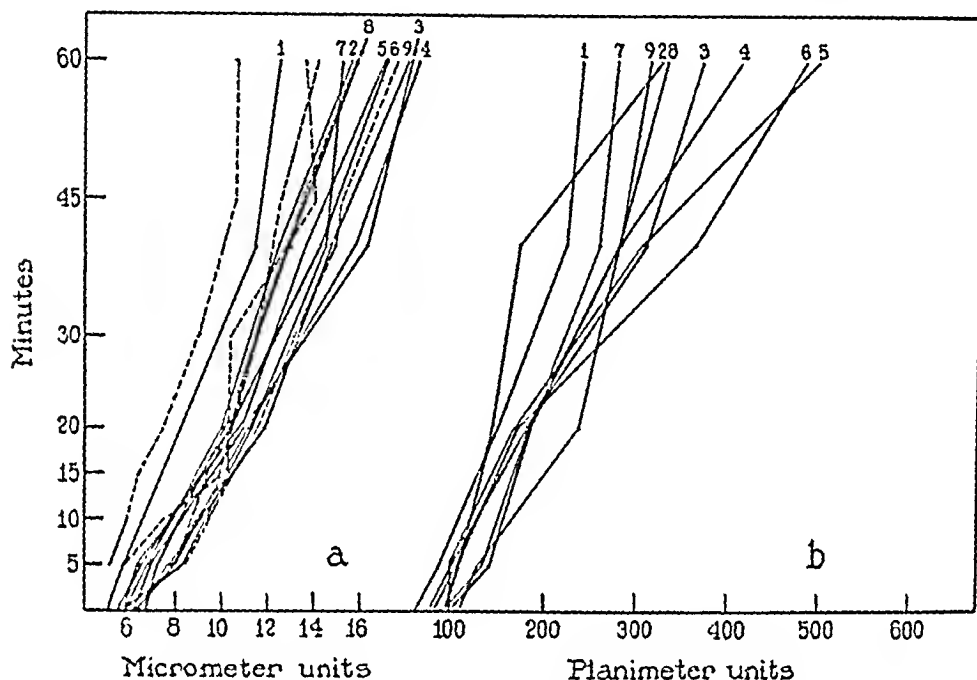
TEXT-FIGS. 2 a and 2 b. The spread of dye in the ears of normal mice anesthetized with luminal.

Text-figs. 2 a, 3 a, . . . 8 a, inclusive depict the spread of dye maculae in the ears of mice during periods of 1 hour, under the varying conditions signified by the legends. The spread has been determined by measurements of the transverse and longitudinal diameters of the spots, through an ocular micrometer scale. It is plotted in units of the micrometer scale as described in the text. The spread of "standard micro maculae" is shown by the continuous lines, that of other maculae by the dotted lines.

Text-figs. 2 b, 3 b, . . . 8 b, inclusive show measurements of the areas of the same standard micro maculae, the spread of which has been plotted in terms of increase in diameters in the corresponding charts of the a series. The areas of the dye spots were determined with a planimeter and have been plotted in planimeter units as described in the text.

Text-figs. 9 and 10 show the spread of dye maculae under the conditions described in the text for each respectively. Spread was determined as in the instances shown in the a series, and is plotted in units of the micrometer scale.

The Effect of Hyperemia on the Spread of Dye through the Tissues.— The depth of anesthesia varied much in the different mice, although the dose of anesthetic was the same. The maculae of dye in the ears of the animals lightly under anesthetic, and responsive to the experimental insult, spread more rapidly than in those of the animals deeply under, and unresponsive. In the former a reflex vasodilatation in the ear occurred promptly after the first prick of the micro needle, the circulating blood was brighter and the blood pressure probably higher. In the deeply anesthetized animals there was no obvious vascular response to the experimental manipulations.

A few experiments were done to test the effect of the reflex hyperemia upon the spread of dye through the tissues.

Other work done in this laboratory for a different purpose has shown that the circulation in the ears of etherized mice is far more active than in those receiving luminal. Consequently to obtain hyperemic ears mice were given just enough ether to keep them quiet during the experimental procedures. The ears of these animals were pink, the vessels filled with bright blood, and far more reactive to the puncturing of the epidermis and the instillation of the dye than the ears of luminalized animals. An especially sharp watch was kept for developing edema, and this involved frequent prodding of the ear with a micro needle for signs of microscopic pitting on pressure. All instances showing it were ruled out. Text-figs. 3 *a* and 3 *b* show the spread of maculae of 2 per cent dye solution in the etherized animals. In Text-fig. 3 *a* the spread is expressed in terms of the increasing sums of the diameters of the maculae for a period of one hour, and in Text-fig. 3 *b* in terms of the increasing areas during the same period. The maculae spread on the average 3.1 times their initial size in terms of the diameter measurements, as compared with 2.7 times in the luminalized animals. In terms of area they spread 4.9 times (Text-fig. 3 *b*) instead of 3.9 (Text-fig. 2 *b*). The average radial spread amounted to 13.2 units or 4.0 mm. In many of the experiments edema occurred. The results in these will be considered separately.

The Effect of Movement upon the Spread of Dye through the Tissues.— In the experiments just reported the average spread of dye through the tissues was greater than that observed in luminalized mice. The slope of the charted curves is less steep. The animals, though lightly under ether, were perfectly quiet and the reactive hyperemia was intense, resulting probably in a blood circulation greater than in the animals previously observed, and at a higher pressure. Next, it became a matter of interest to see what would be the spread of the

dye through the tissues of animals that were moving about. An attempt to approach the normal conditions of movement was made in the following manner.

Just enough ether was given to keep the animals quiet while the 2 per cent dye solution was introduced into the skin. Ether was used so that the circulatory effects would be as much as possible like those of the mice in the experiments just described. Stiff pasteboard collars 4 cm. in diameter, were placed about the necks to prevent the ears being scratched or rubbed against the sides of the cages. As soon as the necessary measurements of the dye spots had been made, the ani-
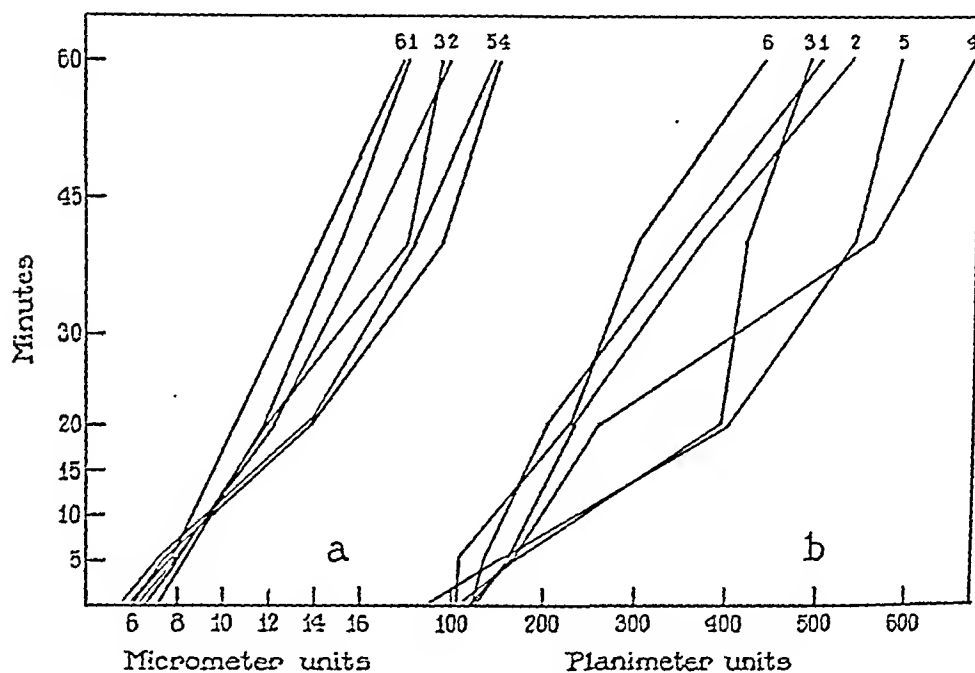
TEXT-FIGS. 3 *a* and 3 *b*. Spread of dye in the hyperemic ears of normal mice lightly anesthetized with ether.

mals were allowed to come out of the anesthetic and within 5 or 10 minutes they were running about the cages in a normal fashion. Further measurements of the maculae of dye were taken at the half hour period and again after an hour with the administration of just enough ether to keep the mice still, while the required observations were made. In Text-figs. 4 *a* and 4 *b* the findings have been charted in the usual way.

In these animals the reactive hyperemia was as great but apparently no greater than that of the mice kept at rest under ether during the entire time of the experiment. A few showed traces of edema of the
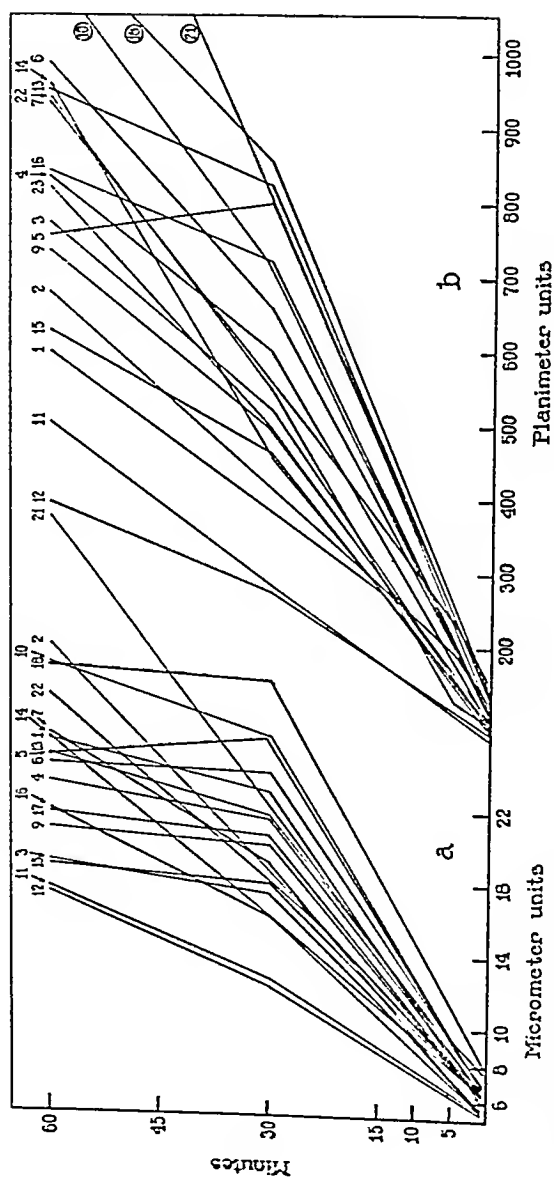
Text-Figs. 4 *a* and 4 *b*. Spread of dye in the ears of unanesthetized normal mice actively moving about.

ear on prodding, though free fluid could never be demonstrated by pricking the skin. These instances were ruled out. The animals varied much in their activity after the spots of dye were placed and the results varied correspondingly from instance to instance. During the first half hour, the dye spread in these relatively normal animals was the greatest that we observed. The average of the measurements of the diameter sums increased 2.9 times after the lapse of 30 minutes and 3.6 times after a full hour. In area the spots of dye increased 5.8 and 8.5 times in the same periods. As the charts show, the slope of the curves in the latter period became much steeper, indicating that the spots were increasing their size more slowly probably because the dye was being absorbed. Table I shows the figures for average radial spread and areal spread. It would seem that normal conditions are optimal for the spread of substances through the tissues and hence for their nourishment. More will be said of this below when other data have been considered.

The Immediate Effects of Hemorrhage upon the Spread of Dye through the Tissues.—It is well known that, following massive hemorrhage, the tissues give up fluid to the blood. Starling (10–12) and Leathes (13) first showed that the fluid shift under these circumstances occurs with great rapidity. As we wished to test the rate of the spread of dye in tissues which were relatively dehydrated, 22 mice of about 30 gm. body weight were bled approximately one-third or one-half their blood volume, that is to say 0.7 to 1.0 cc., by a method now to be detailed.

To 200 cc. of N/10 HCl in a beaker, 1 cc. of the pooled blood of 10 mice, bled for other purposes, was added and the hemoglobin converted to acid hematin. The resulting amber mixture served as a "standard" for the color comparisons which were made in the experiments to be described below. Its hemoglobin content was determined by the method of Newcomer (14) and thereafter the fluid was kept in a beaker sealed with heavy rubber sheets, to prevent evaporation, there being no air space above the fluid. When not in use for color comparisons the fluid was kept in the ice box. All the hemoglobin readings described below were done in a period of about 8 days. Mice of 28 to 30 gm. body weight, anesthetized with luminal, were placed on the plasticine moulds as usual, with the ears resting on porcelain plaques and the tail immersed in 200 cc. of warm N/10 HCl in a beaker similar to that containing the standard solution. The tail was snipped with sharp scissors and blood allowed to collect in the beaker until the

rapidly stirred contents assumed a color like that of the standard.   In this manner, approximately 1 cc. of blood was removed from each mouse in 4 to 8 minutes. The fluids so obtained were placed in closed containers to prevent evaporation and an hour later, after the hemoglobin had been given ample time to be converted to acid hematin, their colors were compared with that of the standard in the Dubosq colorimeter and read together with the latter against Newcomer's glass standard (14).   If the hemoglobin reading of the sample taken from the bled animal showed that less than approximately 0.7 cc. of blood had been taken, the experiment was discarded.
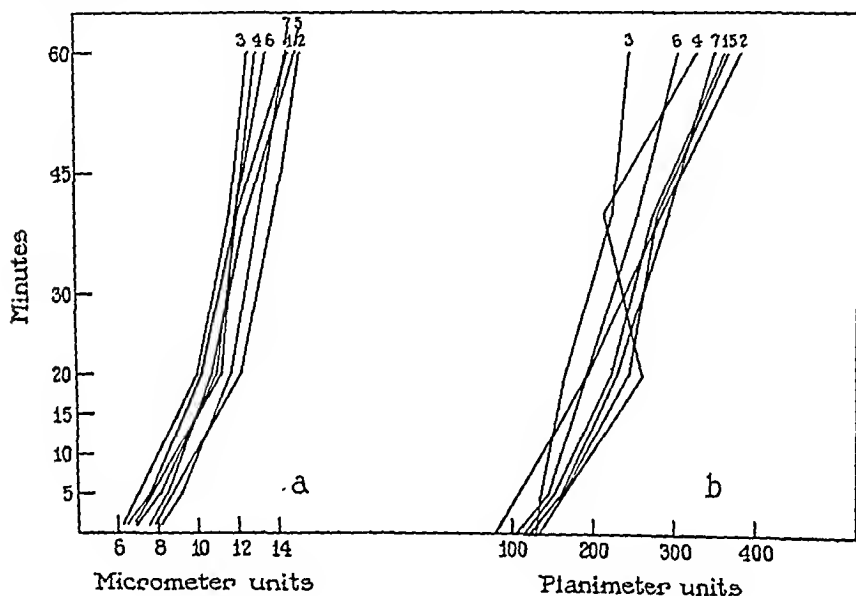
TEXT-FIGS. 5 a and 5 b. Spread of dye in the ears of mice after severe hemorrhage.

Within a minute after bleeding the animals, maculae of the 2 per cent pontamine sky blue were placed in the ears as usual.   This required less than a minute.

Text-figs. 5 a and 5 b show the spread of 7 standard dye maculae in the ears under these conditions, the first expressing the increase in the sums of the diameters, the second the increase of the areas of the same maculae, numbered similarly in both charts.   An average spread of 2.0 times the initial size, in terms of diameters, and 2.9 times in area was obtained in this experiment, far less than that occurring in the luminalized mice (2.7 and 3.9 times respectively).   The radial

and areal spreads averaged 6.8 units (2.1 mm.) and 218 units (19.2 sq. mm.).

In this experiment despite the severe bleeding the animals had undergone, about half showed a formation of edema of the ears after pricking the skin and inserting the dye. The findings in these instances will be outlined below, together with the findings in the edema occurring under other circumstances.

Dye Spread in the Ears of Dead Mice.—Mice of 20 gm. body weight were given luminal in the usual manner and then killed with ether
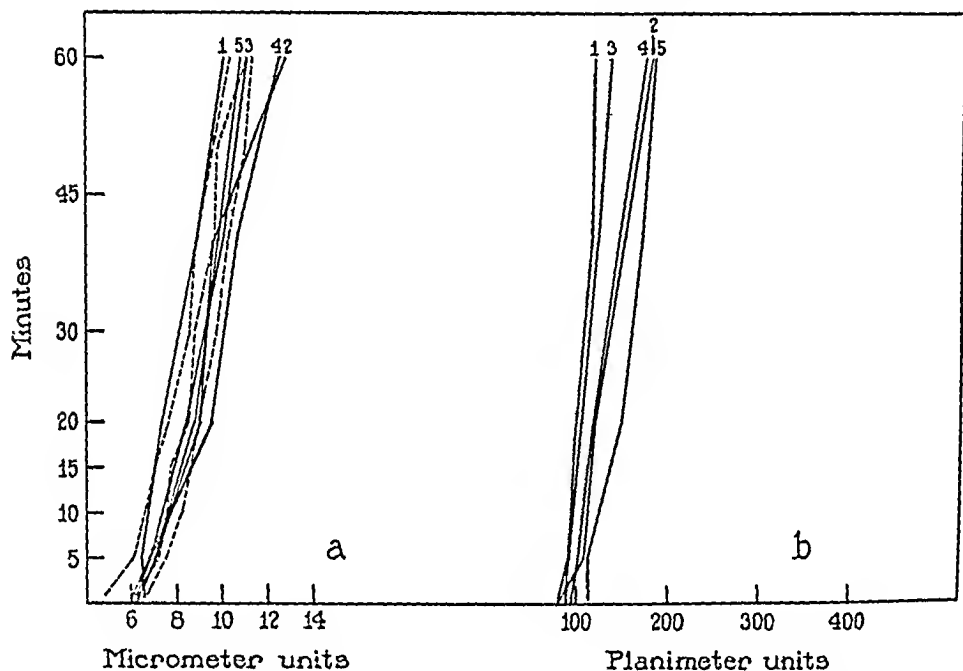
TEXT-FIGS. 6 *a* and 6 *b*. Spread of dye in the ears of mice after death.

or chloroform just before placing maculae of 2 per cent pontamine sky blue in their ears. Under these circumstances there was very little spread of dye in the tissues. Text-figs. 6 *a* and 6 *b* show the spread from 5 standard maculae and 3 others, made with uncalibrated pipettes, (dotted lines) which happened to be the same size. They spread on the average but 1.8 times in diameter sum and 1.8 times in area. The average radial spread of only 5.1 units (1.5 mm.) and the areal spread of 77 units, only 6.7 sq. mm., were the smallest ever observed. The spread of dye through an edematous ear of a dead animal was far greater, as will be demonstrated further on.

The Influence of a Forming Edema upon the Spread of Dye through the Tissues.—As already mentioned, some of the normal mice anesthetized with luminal, and even some of those which were anesthetized with luminal and then bled, developed slight edema in the tissues after instilling dye into the ear. The edema became demonstrable under the binocular microscope by an obvious increase in the thickness of the ear tissue or by pitting of the skin when pressed upon by a micro needle. These instances, excluded from the foregoing comparisons, will now be considered. In all, the edema formed as the dye spread, and became visible about 15 minutes after making the spot of dye. In all the instances too the appearance of edema was preceded by reflex hyperemia which followed the manipulations necessary to form the puncture wound and instil the dye. Whether or not the edema was due in part to the introduction of the dye we do not know. The ears seemed to become more edematous as the period of observation continued.

The rate of dye spread was increased in all, as evidenced by Text-figs. 7 *a* and 7 *b*, and 8 *a* and 8 *b*. Text-figs. 7 *a* and *b* present the measurements of the dye spots in the ears of animals treated as in the experiment from which Text-figs. 2 *a* and 2 *b* were taken, that is to say, in quietly resting mice anesthetized with luminal. In these instances, however, the ears became edematous during the period of observation. The charts show the spread of the dye spots in terms of the sums of the diameters and of their areas, the lines which bear the same numbers corresponding to the same spots, as usual. During an hour the averages of these measurements increased 3.6 and 5.6 times respectively. The spread was far more than that in luminalized mice, when there was no edema. The radial spread of 16.0 units (4.9 mm.) and the areal spread of 404 units (35.8 sq. mm.) show a marked difference too.

In Text-figs. 8 *a* and 8 *b* the measurements of dye spread have been charted for the ears of those bled animals which developed edema despite the hemorrhage. An average increase of 2.7 and 3.4 times is greater than that occurring in the ears of bled animals which did not develop edema (Text-figs. 5 *a* and 5 *b*). The average radial and areal spreads (Table I) are even greater than was observed in the normal ears of quiet animals under luminal.

The findings show that dye spread increased in the ears becoming edematous. Great irregularities appeared owing, no doubt, to the fact that edema occurred in a hit-or-miss manner, the degree of edema differing with the intensity of the reaction of the individual animals.

Because of this irregularity experiments were next planned to study the spread of dye in ears rendered edematous under controlled circumstances.
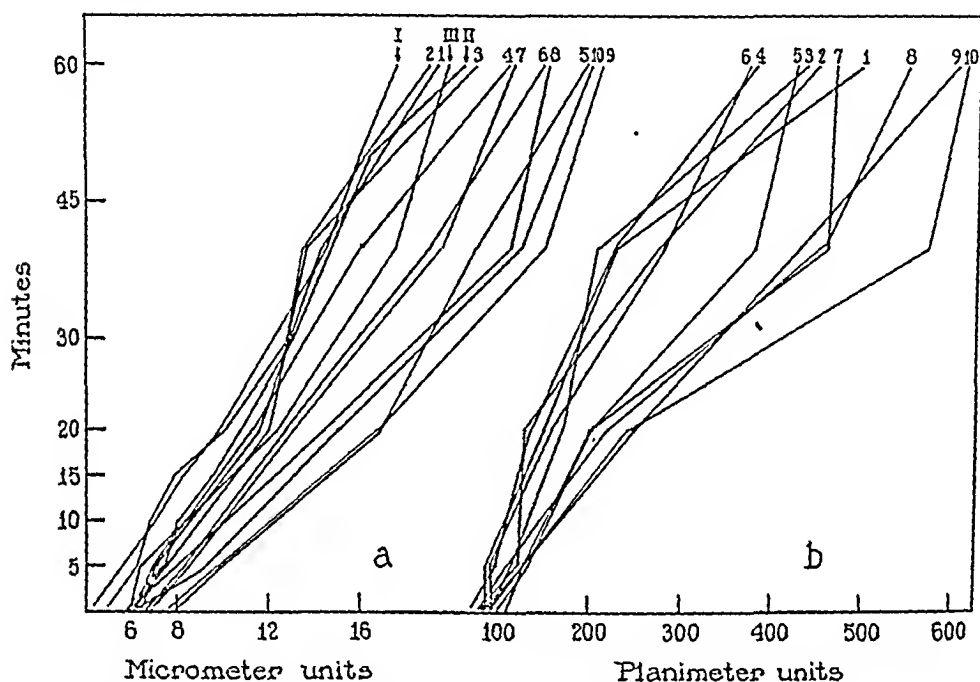
TEXT-FIGS. 7 *a* and 7 *b*. Spread of dye in the ears of normal mice during the formation of edema.

With a camel's hair brush xylol was repeatedly applied to the ears of mice anesthetized with ether. A reactive hyperemia developed almost at once and within a few minutes an intense edema appeared. The ears, which pitted on pressure, assumed a ground-glass appearance under the microscope, and droplets of free fluid escaped when the skin was punctured by the micro needle. Maculae of dye were placed as usual in such ears to study the effect of the rapidly appearing edema.

These experiments were not successful. In almost every instance free fluid began to escape from the puncture wounds within 5 or 10 minutes after the instillation of the dye. The free fluid was deeply colored by the pigment and the maculae spread for only a few minutes

and then rapidly decreased in area as the dye was forced to the surface, or perhaps taken up by the blood, for the ears were intensely hyperemic.

Dye Spread through Tissues Already Edematous.—What can be said of the effect of an excess of fluid already present in a tissue, upon the spread of dye through it? To test the point, maculae of dye were placed in the ears of mice, in the manner described, at intervals from 2 to 18 hours after inducing edema by paintings with xylol. In these
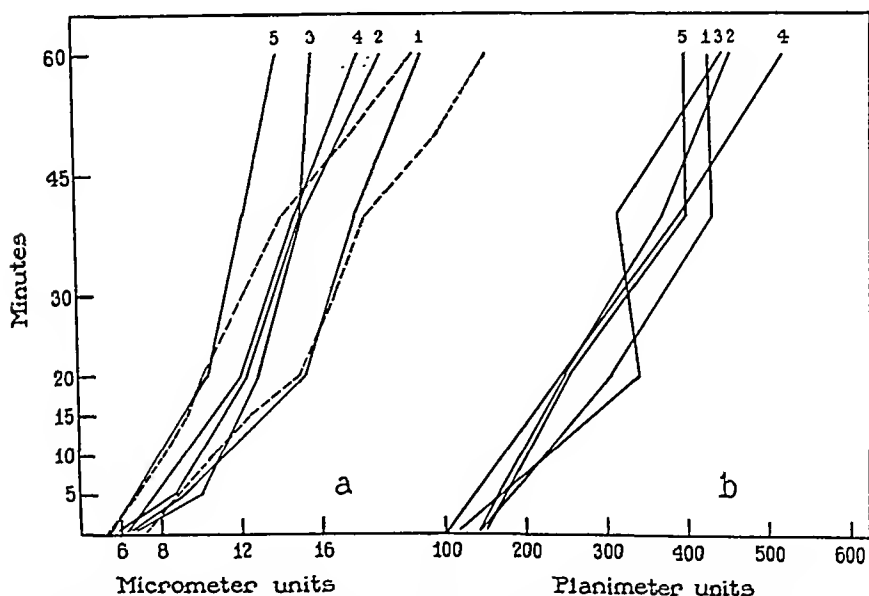
TEXT-FIGS. 8 *a* and 8 *b*. Spread of dye in the ears of mice during the formation of edema but after hemorrhage.

ears, already edematous, the dye often oozed from the puncture wounds and measurements could not be satisfactorily made. However, in some of the experiments this difficulty was not encountered. The spread of these maculae was far less than that of similar dye spots during the formation of edema (Text-figs. 7 *a* and 7 *b*) and was about like that seen in the normal ear. For brevity only one chart is reproduced, Text-fig. 9, to show the spread of those dye spots which were placed in ears 3 to 4 hours after the edema was induced. No oozing of colored fluid took place in these instances. The curves are

plotted in terms of the measurements of the sums of the diameters of the maculae. It will be seen at once that their slope is like that of the curves in Text-fig. 2 *a*. At the end of the experiments the dye spots were, on the average, 2.7 times their initial measurements. The average of the areas, not shown in any chart, increased 4.0 times (Table I). The radial and areal spreads, Table I, were 10.8 units (3.3 mm.) and 297 units (26.1 sq. mm.) respectively. Clearly the
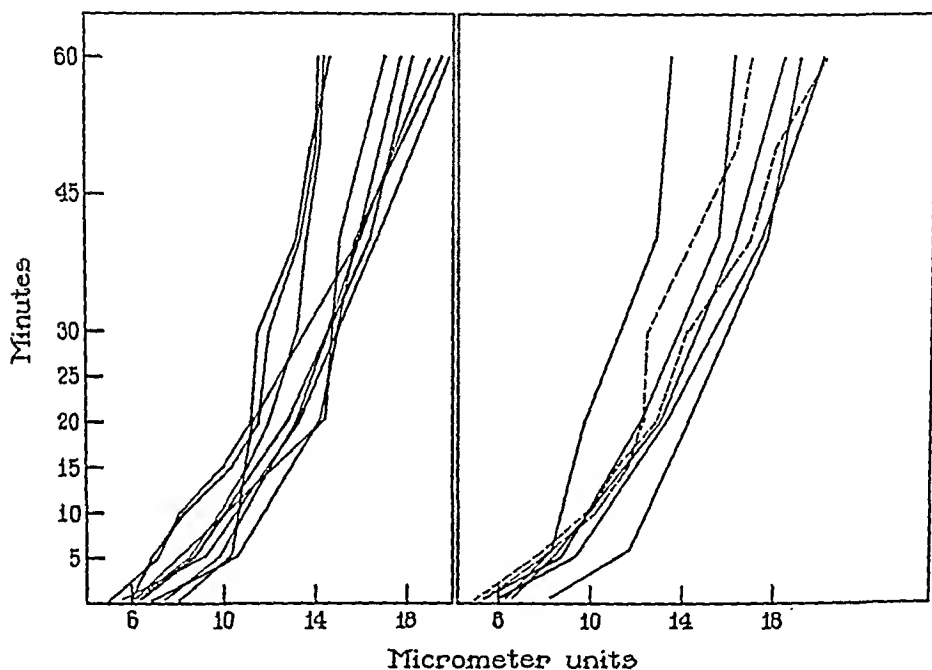
TEXT-FIG. 9.   TEXT-FIG. 10.

TEXT-FIG. 9. Spread of dye in the edematous ears of mice after the edema had formed.

TEXT-FIG. 10. Spread of dye in the edematous ears of mice killed after the edema had formed.

presence of an edema already formed did nothing to speed up the spread of substances through the tissue even though free fluid was present in excess, as shown by the frequent appearance of droplets of fluid at the surface of the minute puncture wounds. This finding will be fully discussed in a following paper after presenting further data which bear upon the theme.

The Spread of Dye through the Edematous Tissues of Dead Animals.— The ears of mice were painted with xylol as in the preceding experi-

ments and the animals killed 3 hours later, after the edema had become intense. Spots of dye were placed in the ears in the usual way. In Text-fig. 10 we have plotted the spread of these spots as determined by their diameter measurements. The slope of the curves is very similar to those of Text-fig. 9, showing that dye spreads with equal rapidity in the ears of dead and living animals when edema of the ears is present. The figures given in Table I bear this out, too. Dye spread was much faster than in the non-edematous ears of dead animals, Text-fig. 6 *a*, and closely approached that observed in normal living ears, as a comparison with Text-fig. 2 *a* and a reference to Table I will show.

The Effect of Mechanical Stresses upon the Spread of Substances through the Tissues

In actively moving unanesthetized animals the dye spread through the tissues with great rapidity. For this reason it seemed wise to test the effect of slight changes in external pressure upon dye spread.

The ears of 20 mice were subjected to mild intermittent changes in external pressure after maculae of dye had been placed in them. The ears lay upon a soft rubber tambour and beneath a glass cover slip while pressure changes were exerted by the tambour 20 times a minute. Pressures of only 2 to 8 cm. of water were used.

By fixing thin rubber tissue over the end of a glass tube 3 mm. in diameter and connected by rubber tubing to a levelling bulb which could be mechanically raised or lowered at will, a small tambour was constructed. The glass tube was filled with water, the remainder of the apparatus with mercury. After forming dye maculae in the usual manner, the ears of mice were placed over the tambour, between it and a glass cover slip, in such a way that dye spots could be observed under the microscope. Intermittent pressures, equivalent to a column of water 2 to 8 cm. of water in height, were brought to bear upon the ear by the tambour. Each period of pressure endured but 1 second, the periods of relaxion, 2 seconds.

The mild pressure changes produced an enormous increase in the rate and extent of the dye spread. The color extended through the ears so far and so fast that repeated measurements could not long be made, for the margins of the maculae became too indistinct. In many instances, after 2 or 3 minutes of application of the intermittent pressure the whole ear became colored by the dye. Slight changes

in external pressure, then, caused far greater differences in dye spread than any of the other factors studied. This finding, too, will be discussed below.

<div align="center">DISCUSSION</div>

The spread of dye in a tissue is subject to many influences. Our experiments have given full play to some of these and ruled out others, demonstrating to what degree they enhance or hinder spread. The greatest spread of dye appeared in the ears which were subjected to intermittent changes in external pressure, the next greatest in those of relatively normal, actively moving animals. In resting, quiet mice the spread of color was most pronounced in those previously normal ears which became frankly edematous during the period of dye spread. It was almost as great in the hyperemic ears of etherized mice. It was less in ears frankly edematous to begin with, and in the ears of normal luminalized mice, but much less in the dehydrated tissues of bled animals and least in the non-edematous ears of recently killed animals.

Histological studies of the tissue changes incident to edema in the ear of the mouse have been reported by Pullinger and Florey (15). As a result of the collection of fluid the connective tissue fibers and other formed elements of the ear are widely separated. In our experiments, puncture of the skin of edematous ears of either living or dead mice led to the appearance of droplets of free fluid at the surface of the organ, from which it seems probable that the wide spaces of the edematous ear are filled with free fluid. The spread of dye taking place through living ears that are already edematous and boggy before the dye is introduced (Text-fig. 9) is about like that taking place in the edematous ears of dead animals in which there can be no fluid movement. The spread in the latter instances must represent that consequent on diffusion alone through the free fluid in the tissues of the ear. From this one can infer that in marked edema in the living animal diffusion is the chief factor in extravascular spread.

In the non-edematous ear of the dead animal spread of dye is very slight, from which one can infer that the main factors responsible for spread in normal ears cease to act after the circulation has ceased. There must be mechanisms operative in the normal living ear which in some way overcome the barriers to spread.

In some of the experiments maculae of dye were placed in normal ears which, during the hour period of observation, became edematous. In other experiments edema and free fluid were already present in the tissues before the dye was introduced.   Dye spread was increased under the first set of circumstances and not under the second.   In the ears of those animals in which edema occurred after the spots of dye had been made, there were periods of a few minutes during which fluid was collecting imperceptibly between the formed elements or connective tissue fibers.   Something evidently happened to increase the spread of dye through the tissues during those periods before one could recognize edema, and while the cells and connective tissue fibers were still in close approximation or forced apart but slightly by the increasing tissue fluid.   That is to say the increase in the rate of dye spread occurred before the tissues became boggy, before the formed elements became widely separated by fluid and edema became visible.

The spread of dye along or between tissue elements not widely separated should be favored by any mechanical forces which tend to rub or squeeze the formed elements together.   As shown above, slight intermittent changes in external pressure have this effect and greatly increase the spread of dye through the tissues of normal ears.   A similar mechanical effect by the pulsation of blood vessels has been demonstrated in preceding work (1).   One would expect the pulse, acting in this way, to increase the spread of the dye through the tissues only during the period in which an edema is forming, for when the formed elements have become widely separated by fluid, their movements will no longer tend to squeeze fluid from between them.   The experiments reported here favor this point of view for, as just described, it actually has been observed that dye spread is not as great in a quiet, edematous tissue after it has become boggy, as in a tissue just beginning to become edematous.   Further discussion of this problem must be deferred to the following paper, which reports data on the manner of the interstitial movement of substances.

SUMMARY

A method has been devised to measure the spread of vital dyes in the skin of mice.   Spread is greatly influenced by physiological and pathological changes which add fluid to the tissue or abstract it.

Spread is greater in the quiet, living ear than in the ear of an animal just killed. It is equally considerable in the frankly edematous ears of living and dead animals, and not greater in either case than in normal, quiet tissues. During the early stages of edema formation on the other hand, dye spread is notably rapid. It is still greater in the ears of normal animals actively moving about, and is greatest in tissues subjected to very gently intermittent changes in external pressure.

The significance of these findings is discussed.

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A STUDY OF HUMAN SKIN GRAFTED UPON THE CHORIO-ALLANTOIS OF CHICK EMBRYOS*

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PLATES 47 TO 49

The possibility of successfully grafting certain malignant tumors of the rat and mouse upon the chorio-allantois of chick and duck embryos was demonstrated by Murphy (1) in 1912. Heterologous grafts of this kind became embedded and vascularized, and grew rapidly and continuously under these conditions until about the 18th or 19th day of incubation. At that time there seemed to be a sudden change whereby the grafts were caused to undergo rapid regression and degeneration.

It seems likely that the chick embryo, up to the last days of incubation at least, cannot respond as adult animals do to the presence of heterologous tissue, and in the absence of such unfavorable reaction the foreign cells are capable of utilizing the nutriment supplied by the developing egg.

The capacity to respond in a way which is unfavorable to heterologous and iso-grafts probably develops gradually in the chick; for the experiments of Danforth and Foster (2) have shown that during the first few days after hatching iso-grafting can still be successfully performed upon them.

Owing to the demonstration of successful hetero-grafts of certain tumors and embryonic tissues upon the chorio-allantois of the chick, and to the demonstration that the chick membrane when subjected

to injury by heat would form granulation tissue, the possibility occurred to us that human skin might be nourished when suitably applied to this highly vascular embryonic tissue. We felt that the study of the fate of such grafts would be of interest in itself, and successful grafts would afford an opportunity for an experimental approach to a variety of problems.

Our experience readily demonstrated the possibility of securing grafts of human and other animal (rabbit, chicken) skin upon the chorio-allantois of chick and duck embryos. Such grafts rapidly became fixed and nourished so that they persisted intact until about the time of hatching. Grafts implanted upon the membrane of 10 day old embryos could be studied for a period of 8 to 10 days, the incubation period of hen eggs being 21 days.

The present paper concerns the technique used, and records our observations upon the mechanisms involved in the survival of grafts of human skin upon the chorio-allantois of chick embryos. It includes also a discussion of certain principles and possible applications of the method.

Technique

Fertile hen eggs were incubated in a commercial hatchery incubator for 10 days. By candling, the large membranal vessels were located and marked on the shell, because it seemed that grafts survived better when placed over them. A square window was then cut in the shell by the use of a rotating carborundum disc according to the method described by Goodpasture and Buddingh (3); and a piece of skin, previously prepared, was applied to the exposed chorio-allantois. The raw corium of the graft was affixed to the presenting vascular ectodermal layer of the membrane; thus the epithelium of the skin presented exteriorly upon the surface as in its natural relationship.

Human skin in three forms was received immediately from the operating rooms of the Surgical Service of the Vanderbilt Hospital. This had been removed in the course of excising tumors or in covering raw surfaces with skin grafts. Pieces of skin, which were trimmed away in fitting various grafts in their beds, were saved and sent to the laboratory. Other pieces surrounding various lesions of the skin were received with the subcutaneous tissue still attached. Additional specimens were of the thickness of thin grafts (Thiersch grafts), while still others were a little thicker but still not of the full thickness variety (split grafts).

The intact skin had been previously shaved and washed with soap, water and alcohol, on the ward, followed by iodine and alcohol in the operating room in preparation for removal. The pieces were stretched upon a sterile board and cut

into squares of ½ to 1 cm., or larger if desired.   Each of these was then spread, with the raw corium exposed, upon the smooth surface of a thick metal searing iron having a long handle.   A dissecting needle was used to effect these manipulations.   By means of the iron the flattened and stretched surface of the corium was directly applied to the chorio-allantois, where it was freed with the needle and laid upon the membrane.   Any folded edges or corners were straightened out with no difficulty.   The thin graft then lay smooth and flat upon the membrane to which it rapidly became adherent.   These manipulations were of course carried out with precautions for asepsis.   If skin with subcutaneous tissues attached was received it was stretched and fixed to the board with pins and thin pieces of epithelium with a little corium were shaved off with a triangular fragment of a safety razor blade held in a hemostat.   Full thickness skin was never used for grafting.

After the graft was thus affixed to the membrane the window was closed by surrounding the shell opening with a layer of vaseline-paraffin mixture upon which a cover-glass was layed and sealed.   The egg was then placed on a wire meshed tray in a bacteriological incubator held at 37°C. and kept moderately moist by means of a vessel of water.   The eggs were placed in the tray with the window up.

Through this window the graft was observed at will either with the naked eye or through a binocular microscope.   Moisture under the cover-glass was removed by applying to the outer surface a heated, blunt instrument, like the tip of a scalpel handle.

The graft usually remained smooth and flat for 25 to 72 hours, then it tended to become somewhat wrinkled with a diminution in circumference.   The wrinkling was probably due to contraction of the proliferating mesodermal tissues of the membrane.

If the graft was successful it remained firm and glistening.   If necrosis occurred it became boggy, dull and yellow.   Surprisingly rarely did the surrounding membrane become infected or inflamed.   At times staphylococci grew upon the keratinized surface of the graft without interfering in any way with its nourishment and survival.   If such grafts are clean, thin, fresh and only slightly traumatized, the chances of survival are excellent.

At variable periods of time the membrane with its graft was fixed in Zenker's fluid; and paraffin sections were cut and stained, in order to observe the processes of nourishment of the grafts.   The membranal blood vessels in several instances were injected with a 10 per cent suspension of Higgins' India ink, and after fixation, paraffin sections were studied in order to observe the vascular bed.

Attempts were made to study the degree of reestablishment of circulation in the peripheral vessels by direct observation with a Leitz binocular ultropak microscope.   Grafts at various stages were observed through the uncovered window. Oils and other substances were applied to the epithelium to make it more transparent.   So far, however, we have not been sufficiently certain of our observations to include them.

Structure of the Chorio-Allantoic Membrane of Chick Embryos

At 10 or 12 days of incubation, as Danchakoff (4) has shown, the exterior or ectodermal layer of the chorio-allantois is a thin, highly vascular membrane in which a rich capillary network lies beneath and within the delicate epithelial sheet. Some of the capillary loops seem to be exposed upon the surface without epithelial covering. It is through this membrane that the respiration of the embryo takes place.

On removal of the shell and its adherent fibrous membrane, there is sometimes slight injury to the ectodermal-capillary layer and minute extravasations of blood occur onto the surface; so it seems certain that either because of its original structure or because of slight damage some points of the graft were placed directly in contact with the exposed capillary bed. In effect, therefore, the grafts lay upon a vascular surface analogous to granulation tissue.

Survival of the Grafts and the Processes of Their Organization

Very soon after the application of a piece of skin to the membrane the graft becomes lightly adherent. Microscopic sections at the end of 24 hours show no exudate between it and the membrane, neither fibrin, serum nor leucocytes, if the graft is an entirely satisfactory one. Even at this time, however, there may be wide or narrow areas of the membrane which are entirely denuded of epithelium, and at these places the corium of the graft is closely applied to the embryonic mesodermal tissue. At other places the ectodermal layer of the membrane may be more or less intact, possibly with occasional microscopic petechiae between it and the corium. Frequently chick polymorphonuclear leucocytes have infiltrated parts of the graft.

At 48 hours one may occasionally see a capillary protruding through the ectoderm of the membrane beneath the corium. As early as the 3rd or 4th day nucleated erythrocytes of the embryo have found their way into preexisting blood vessels of the skin graft, and at times on the 5th day every blood vessel of the graft, even the subepithelial capillaries, is filled with the chick erythrocytes, giving the appearance of a complete establishment of blood circulation through the vascular bed of the skin. This appearance has been particularly marked in grafts from keloids and moles in which the corium is more rigid than in normal skin, and the blood vessels larger. If a graft "takes" satisfactorily there may be little or no cellular exudate to be found in it, and after 5 days sections have been obtained which contain no cellular exudate whatsoever.

In those grafts or parts of grafts which do not take satisfactorily polymorphonuclear cell and serous exudates appear early and abundantly in the corium,

and may increase until the entire graft or its epithelium has become elevated by a pustule or a vesicle. These usually die in part or altogether in a few days, although some portions of such grafts may become adequately nourished.

In autografting on human subjects considerable stress has been placed upon the importance of obtaining and maintaining contact of the graft with the underlying wound surface, especially during the first few days after its application. An exudate is prevented from collecting under the graft either by cutting the latter in pieces so small that drainage may occur from under its edges ("pinch grafts") or by perforating the larger grafts at frequent intervals for drainage and maintaining pressure over its surface by a marine sponge or another expansile medium.

In the grafts on the chick membrane an attempt was made to gain approximation by smoothing the skin out but there was no effort to maintain contact. It is probable that in some of the grafts, especially the larger ones, spaces containing plasma or air remained and that this was the cause of permanent collections of exudate and death of these grafts.

On about the 3rd day evidence is often seen of an ingrowth of capillaries from the membrane into the adjoining layer of the corium. These invading vessels may penetrate deeply into the corium during the 10 days of observation.

If the graft is rather thick and nucleated red cells do not early appear within the lumens of the blood vessels of the graft, evidences of inflammation appear and the corium becomes edematous followed by desquamation or necrosis of the overlying epithelium.

Mitotic figures sometimes increase in number in the epithelium of a satisfactory graft at about the 6th day, and hyperkeratosis becomes evident later. Rarely does one find a mitotic figure in a cell of the corium during the period of observation. There is no tendency for the epithelium of the graft to invade the corium.

The Ectodermal Epithelium of the Membrane in Relation to the Graft

One of the most interesting phenomena in connection with a skin graft on the membrane is the perfect approximation of the ectodermal epithelium of the membrane with that of the piece of skin at its periphery. The fusion is so exact and the union so nearly perfect that it would be difficult to detect it, did the smaller chick epithelial cells not take a lighter stain. In some preparations it appears that the two types of epithelium are laced together by cellular fibrils which pass between them producing intercellular bridges or prickles. One gains the impression of an affinity or positive chemotaxis between the epithelium of the host and that of the cut edge of the graft.

On the other hand an opposite relationship seems to exist between the mesodermal tissue of the corium of the graft and the ectoderm of the membrane. In the presence of the graft the latter tends to

disappear from beneath or to aggregate in small foci where it assumes the appearance eventually of epithelial pearls.

In the case of skin grafts from an old man over 70 years of age the cells of such epithelial pearls underwent rapid multiplication with extensive keratinization so that fairly large dermoid cysts were formed. Our observations of this are too limited to draw definite conclusions, but it is suggested that hyperkeratosis of the graft may influence keratosis of membranal ectoderm.

The exact mechanism by which membranal ectoderm disappears or draws apart in the presence of skin grafts has not been determined. It is of interest, however, that mesodermal grafts (*i.e.* of muscle, spleen and cartilage) rapidly sink into the mesoderm of the membrane and the ectodermal layer becomes completely restored and reunited over them.

The observation was made that skin grafts from two human cadavers also tended to sink into the mesoderm of the membrane, but apparently were prevented from becoming completely incorporated, by a union of the epithelial edges with the ectoderm of the host.

Two skin grafts implanted on the same membrane with edges only a few millimeters apart have shown no tendency of the epithelium to grow over the gap in a period of a week. As in single grafts the epithelial margins were sealed by the fusion with them of the ectoderm of the membrane.

The Membranal Fibrous Tissue in Relation to the Graft

In the case of a well nourished graft wherever the collagen and fibroblasts of the corium have come into contact with exposed mesodermal fibrous tissue of the host, a fusion or interlacing of collagenous fibrils and fibroblasts of the graft and host has taken place. There is no separation by fibrin, hemorrhage or exudate under favorable circumstances. There seems to be as much congeniality between these tissues of the graft and host as is observed between the two epithelia.

Considerable hyperplasia of membranal fibrous tissues sometimes occurs beneath a skin graft, and fibroblasts rapidly extend into areas of inflammation or degeneration in the corium and along the edges beneath the epithelium of the graft accompanied by membranal blood vessels.

Without infection there is usually little or no myeloid hyperplasia

in the membranal mesoderm, and the polymorphonuclear leucocytes which pour out of the blood vessels quickly migrate beneath or into the corium of the graft.

Foreign bodies or exudate become surrounded or walled off by foreign body giant cells which form rapidly and in great abundance.

The Blood Vessels of the Membrane in Relation to the Graft

The earliest evidences of an effect of the skin graft upon the underlying capillaries of the membrane are a denuding of the ectodermal epithelium, and minute focal extravasations of nucleated erythrocytes beneath the graft. In some preparations within 48 hours capillaries may be found penetrating the approximating surface of the corium.

Within 3 or 4 days nucleated chick red cells can be seen inside the lumens of the larger blood vessels of the graft. In many places small extravasations of blood seem to intervene between these vessels and the membranal capillaries. In other preparations, especially in keloid grafts, the two vascular beds seem to join directly, the blood passing immediately from the enlarged membranal channels into the preexisting channels of the graft. The latter become filled with chick nucleated erythrocytes, even to the small subepithelial capillaries, and the channels in serial sections appear to be connected and looped in such a way that at least a partial circulation seems inevitable.

There is no doubt that these channels are the original vessels of the graft, for as late as 5 days serial sections have shown persisting non-nucleated human red cells in capillaries continuous with larger channels filled entirely with chick red cells. Furthermore the endothelial lining of the vessels of the graft are preserved, and the human endothelial cells are larger and possess larger and more chromatic nuclei than do those of the chick embryo.

It would appear therefore that the vascular connections between the graft and the host exhibit also the effect of a certain affinity or chemotaxis which causes them to unite, as do the respective epithelial cells and fibroblasts.

So far as representing a restoration of blood circulation is concerned the filling of the blood vessels of the graft with nucleated red blood cells of the embryo may be deceptive. It has been stated that, in serial sections at a 5 day period of a piece of skin graft, capillaries

were found containing human erythrocytes in continuity with larger channels filled with nucleated cells. The presence of human erythrocytes definitely indicates a lack of circulation through these particular channels, and several attempts to demonstrate blood circulation through the persisting vessels of a graft (not keloid nor mole) by India ink injections have failed to demonstrate it. Arterioles and arteriolar capillaries of the membrane and granulation tissue growing into the corium become filled with carbon particles, but adjacent large channels of the skin, although distended by nucleated chick erythrocytes, contain none.

·The presence of nucleated red cells of the embryo within the vessels of the graft demonstrates access of embryonic blood to them; and this no doubt provides an adequate plasma circulation even though blood circulation is wanting.

It would seem that a true circulation through the original blood vessels of the graft might in time become established if the incubation period were prolonged and the embryo developed no unfavorable reaction. Under such circumstances no doubt chick endothelium would eventually replace the original lining, for there is evidence in older grafts that the human endothelium gradually disintegrates.

No evidence was found within the period of our observations that capillaries from the membrane grow into the preexisting blood vessels of the graft. However there is a constant increase in capillary, arterial and venous ingrowth from the membrane into the corium of the skin, and in all probability this new vascular organization would eventually take over the circulation which initially is maintained in part through the original channels.

There is no doubt that, in the first days of the graft and probably in part throughout the period of our observations, a plasma circulation of some sort is largely responsible for the survival and maintenance of the grafts. If a piece of skin is very thin, consisting almost entirely of epithelium, no vascularization may appear, yet the graft remains to all appearances in perfect condition. Such grafts indeed are less apt to show evidences of inflammation and degeneration than thicker ones.

Early exudation into the graft, however, seems to indicate injury or degeneration of some sort within it before it is applied. To such injury of the foreign tissue the embryo responds by an outpouring of polymorphonuclear leucocytes and fluid exudate.

Beneath the graft the embryonic blood vessels in a few days increase in size and abundance, evidently in response to influences, perhaps both mechanical and chemical, through fluid exchange, from the applied skin.

Chick red blood cells do not penetrate all the vessels of the graft, and in some instances most of the vessels contain none. Under these conditions there is usually considerable invasion by polymorphonuclear leucocytes and the skin appears to be less well nourished. The access of chick blood to the vascular channels of the graft is a very important element in its nourishment for several days, and grafts from keloids and moles in which vascular channels are larger and more easily penetrated take better apparently than do most specimens of normal skin.

In the keloid grafts especially there are prominent endothelial lined lymphatic channels containing human mononuclear phagocytic cells. Not infrequently mitotic figures are found in these cells. No chick erythrocytes have been observed in lymphatic vessels.

Reimplantation of Skin Grafts

Some attempts were made to remove grafted skin from the first implantations and to regraft them upon a second embryo. This was found to be a rather difficult procedure and the attempts usually resulted in failure, although a few successful second generation grafts were obtained. With the development of a better technique the percentage of successful second grafts no doubt would be greater. The difficulty technically lies in removing the graft, which may be wrinkled, in such a way as to avoid carrying over too much embryonic tissue. The primary graft perhaps also lends itself better to a plasmatic circulation than the secondary, because of its intact blood vessels. Infection also is a common cause of failure of reimplantations.

Grafts have been maintained for periods of 8 days on the first embryo and 6 on the second, before sections were made. These 14 day old grafts show extensive keratinization and active mitosis of cutaneous epithelium. Occasional mitotic figures are also found in fibroblasts of the corium, and in endothelial cells. Human skin grafts can be maintained for at least 2 weeks in this way and probably longer.

The possibility of preserving human skin and other tissues for

purposes of surgical use is obvious, although no demonstration of th
practicability of such a method has been made.

Duration of Viability of Skin before Grafting

At present tests are in progress to determine the practicability o
preserving skin and other tissues by this method and regrafting then
to the same and other human individuals. An attempt is thus bein;
made to determine whether or not those individual hereditary factor
or characteristics which are responsible for the failure of iso- or homo
grafts are retained by the tissue after a sojourn of several days ot
the egg membrane.

We have not directed especial attention to the problem of th(
duration of viability of skin for grafting, but in the course of ou:
experiments skin removed from the patient 8 hours before grafting
and kept moist at room temperature (22°C.) has yielded successfu
grafts. In one test human skin kept refrigerated at 5°C. for 24 hour:
gave excellent takes.

In two instances skin removed from cadavers 2 and 3 hours aftei
death was successfully grafted on chick membranes.

Use of the Method in Experimental Pathology

There are probably many uses to which this method of skin grafting
might be put in various experimental studies. Some of our own
interests have been directed toward the field of infection and im-
munity, especially infections caused by viruses.

In order to determine whether or not human skin grafts on the
embryo are susceptible to infection by some of these agents, we have
successfully inoculated human skin grafts with the viruses of herpes
simplex, vaccinia and smallpox. The resulting lesions and cellular
changes have been typical of these infections. These studies are
being continued and will be reported upon later. The question of
cellular immunity is also under investigation.

DISCUSSION

The laws governing the fate of skin when transplanted from one
part of the body to another of the same individual and from one
individual to another are of great interest alike to the pathologist
and to the surgeon.

The experimental work to establish these laws, as far as it has

gone, has met with many difficulties. In human tests the chief difficulty has been that, in order to get complete histological studies at different stages after transplantation, too much skin would need to be sacrificed. In dealing with animals the great amount of hair usually present in the skin makes dressings difficult and infections common when grafting is attempted. If no dressing is applied injury to the surface from mechanical trauma or drying is common and infection will usually ensue.

Davis and Traut (5) avoided these factors to a large extent in their experimental studies on dogs by burying skin grafts, raw surface down, on muscle, and closing the wound edges over them. The grafts were excised later for study. By this method they were able accurately to determine histologically the mode and degree of the reestablishment of circulation at varying intervals after grafting.

Those who have studied the processes involved have recognized three periods in the permanent establishment of mammalian skin grafts which may be enumerated and discussed in relation to our observations as follows:

1. The Stage of So Called Plasmatic Circulation.—This term was first used by Goldmann who was so impressed by the rapid inwandering of mononuclear leucocytes from the host into the vessels and tissues of the corium of the skin, that he felt quite sure the graft, during the first hours of its existence, must be nourished by a sort of circulation of fluids before vascular connections became established (6).

Such a plasmatic circulation seems to be the most important mechanism in the maintenance of human skin grafts on the chick membrane not only in the early stages but throughout the longest period (10 days) of observation.

Unlike the mammalian experiments however, there is in a successful graft only moderate cellular infiltration, and then the invading cells are polymorphonuclear leucocytes. This occurs especially in unfavorable grafts and in and about necrotic blood cells within vascular channels of the graft which are not penetrated by chick erythrocytes, and it seems to be a response to injury and degeneration.

Although there might be an imbibition of fluid directly from the membrane into the corium, the chief nourishment appears to come through a later communication of the two groups of blood vascular channels, whereby first the large and then the small vessels of the

graft become filled with nucleated erythrocytes of the chick, no doubt accompanied by abundant plasma.

2. The Stage of Vascularization of the Graft.—In experiments upon dogs Davis and Traut described, as had others before them, the immediate deposit of a layer of fibrin between the tissue of the host and the graft. Within 24 hours a rich network of capillaries grows into the fibrin layer and begins to penetrate the corium. As early as 22 hours fusion of newly formed capillaries with similar or even larger blood vessels of the graft was demonstrated by the method of injection. This evidence indicated the partial establishment of circulation by anastomosis. In the chick embryo, although there is no comparable newly formed granulation tissue found on the membrane, a rich capillary bed is present in the ectodermal layer of the chorio-allantois. There is abundant evidence that anastomoses occur between the vessels, both large and small, of human skin grafts and the blood vessels of the membrane. Sometimes the two vascular channels seem to be united by an extravascular pool of blood. But there is no conclusive evidence that these connections restore intravascular circulation completely. The process seems rather to be one of inturgescence of the vessels of the graft for the most part.

There is, however, an ingrowth of capillaries from the membrane into the proximal layer of the graft after about 48 hours, but this does not lead to an effective blood circulation during the period of our observations. There is no evidence of a complete revascularization of the graft by chick blood vessels, such as took place in about 8 days in the experiments of Davis and Traut upon dogs. Granulation tissue rich in blood vessels forms especially where the corium of the graft is degenerating or disintegrating. The normal corium is apparently relatively impervious to the ingrowth of chick vessels.

3. The Period of Regeneration and Repair.—As described by Davis and Traut, the degenerative changes in full thickness grafts implanted in dogs are of an extreme degree, resulting in a slough of practically all the epithelium, except the thin Malpighian layer, and extensive degeneration in the corium including necrosis of the endothelial lining of blood vessels.

This does not occur under favorable circumstances in thin grafts of human skin implanted upon the membrane. All layers remain intact and there is no disintegration of epithelium. On the contrary

it remains normal in appearance.   The blood vessels persist and, if the chick blood gains access to them, the endothelium remains intact for several days.   If they are not penetrated by chick blood, the contained human white blood cells and endothelium undergo necrosis and the lumen is invaded by chick polymorphonuclear leucocytes.

No evidence of a revascularization by an ingrowth of capillaries and granulation tissue into the lumens of blood vessels of the graft was seen, such as was described by Davis and Traut in their experiments.

There is a greater tendency in the grafts which we have studied for the corium to undergo degeneration than the epithelium, and, as others have observed, epithelium of hair follicles seems to be the most viable tissue present.

Because there is no primary loss of epithelium through necrosis and sloughing there is no particular period of regeneration.   Growth of epithelium proceeds apparently continuously, although most mitotic figures are evident about the 6th day.   Keratinization ensues, and in grafts 10, and regrafts 14 days old, there is evident hyperkeratosis, for there is no loss from desquamation.

If incompletely nourished the corium tends progressively to disintegrate and there is no period when we have observed regeneration of fibroblasts to any extent.   The corium, however, may remain perfectly intact for a week or longer if chick blood readily gains access to the blood vessels of the graft.

The chorio-allantoic membrane of the chick embryo thus offers a favorable medium for the nourishment of human skin grafts during 10 days, which represents the longest period of our observations. Implants made on the 10th day of incubation will survive intact for at least 10 days without any conclusive evidence of a specific reaction of this host against the heterologous tissue.   The embryo, until about the time of hatching at least, shows no evidence in these experiments of being able to react unfavorably in a specific way to the grafted foreign tissue.

SUMMARY

Human skin grafted upon the chorio-allantoic membrane of chick embryos adheres and becomes nourished for as long as 10 days.

Occasionally regrafts upon a second egg have succeeded and thus prolonged the vitality of the graft to 14 days.

In successful experiments the epithelium of the chorio-allantois fuses with that of the graft, the collagen fibers of the corium interlace with those of the membrane after the separation or disappearance of the ectodermal layer, and the blood vessels of the chick anastomose, and unite by intervening pools of extravasated blood, with those of the graft. This vascular communication between the two tissues is largely responsible for the nourishment of the graft by affording a plasmatic circulation.

Gradually there is a partial revascularization of the graft by an ingrowth of blood vessels from the chick membrane.

Human skin grafts were susceptible to experimental infection by several viruses.

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EXPLANATION OF PLATES

The stains used for the specimens illustrated were hematoxylin and eosin.

PLATE 47

FIG. 1. Graft of human skin on the chorio-allantoic membrane of a hen's egg. Duration of graft 5 days.

FIG. 2. Pigmented mole of human skin grafted 5 days on chick membrane. A and B are epithelial pearls of chorionic epithelium marking position of original ectodermal layer.   × 120.

FIG. 3. Graft of human skin on chick membrane.   Duration 10 days.   × 18.

FIG. 4. Pigmented mole of human skin.   Duration 8 days.   Note blood vessels of membrane enlarged and those of graft engorged with chick erythrocytes.   × 40.

FIG. 5. Graft of human skin, infected and necrotic.   Duration 4 days.   Note zone of inflammatory reaction in membrane.   × 30.
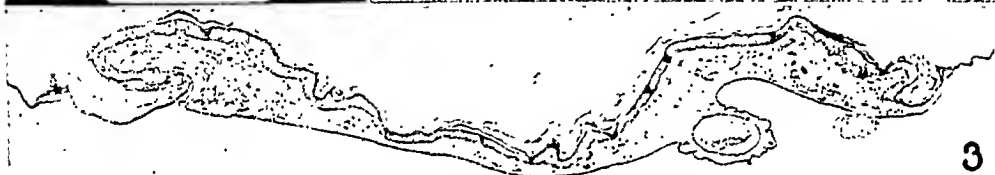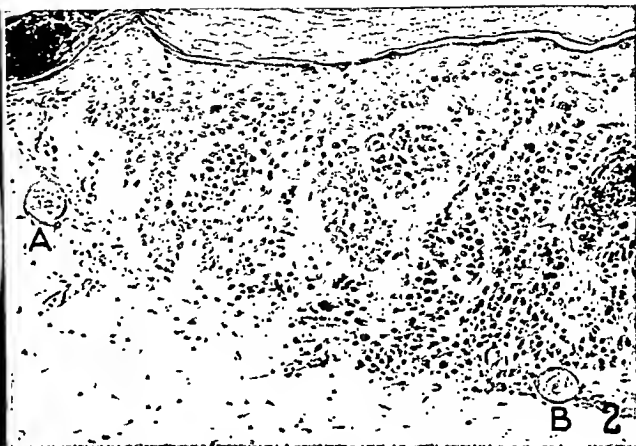
PLATE 48

Fig. 6. Human skin grafted 5 days. Note (A) vessel of membrane communicating with vessel of graft distended with chick erythrocytes (nucleated). × 225.

Fig. 7. Graft of keloid, duration 5 days. Note communication of blood vessels of membrane and graft. × 225.

Fig. 8. Same as 7 showing (A) human erythrocytes and (B) chick erythrocytes in same blood vessel. × 1100.

Fig. 9. Graft of keloid, 5 days. Note (A, B, C) are of apparently communicating blood vessels of graft filled with chick erythrocytes and communicating with enlarged vessel of the membrane (D). × 150.

FIG. 10. Note (A) fusion of membranal ectoderm (left) with epithelium of graft (right). Duration 5 days. × 225.

FIG. 11. Serous and leucocytic exudate in part of graft (8 days) next to a pocket of serum and pus (not shown). × 200.

FIG. 12. Another portion of same graft (Fig. 11) showing good take and no exudate. × 200.

FIG. 13. Granulation tissue from membrane extending into corium of graft between two epithelial pearls of membranal ectoderm. Duration 5 days. × 225.
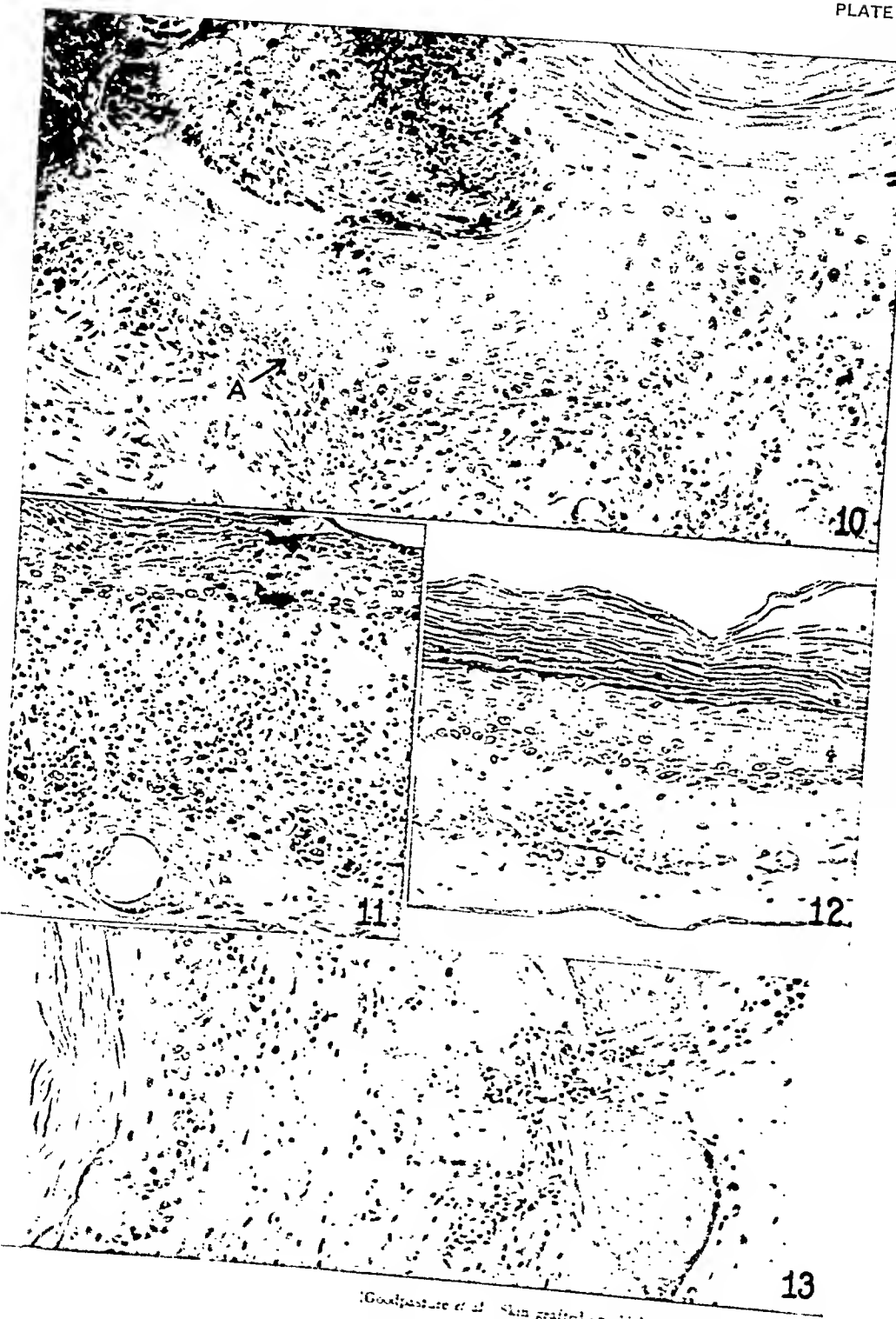
THE SIGNIFICANCE OF HUMAN DOUBLE ZONE BETA HEMOLYTIC STREPTOCOCCI IN THE UDDER OF THE COW

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In 1934 Brown (1) reported the isolation of double zone beta hemolytic streptococci from human sources. He stated that, previous to this, streptococci found producing double zones had been from cow's milk. It has been shown by Lancefield (2), Hare and Colebrook (3), Lancefield and Hare (4), and Brown (5) that some strains of beta hemolytic streptococci (serological group B) from the human throat and vagina resemble closely, both culturally and serologically, certain bovine mastitis organisms.

The purpose of the present study was to determine whether mastitis could be produced in the udder of the young cow by the introduction of double zone beta hemolytic streptococci from human sources, using methods previously reported (6–8). This work was undertaken at the suggestion of Dr. J. Howard Brown, with cultures which he furnished.

EXPERIMENTAL

Animals.—4 young cows, early in their first lactation period, were used. All were kept under good dairy conditions and fed a full production ration. They were milked twice daily, at 9 a.m. and 9 p.m.

Following the first inoculation, the attendants wore rubber gloves while milking, and the uninfected quarters were milked first. When two quarters were involved, one was milked with the right, and the other with the left hand. After the first two quarters had been done, the gloved hands were thoroughly washed in soapy water and dried before the remaining two were milked. When all the quarters had been inoculated, the one treated with the bovine culture was milked after the other three quarters had been emptied.

Laboratory Examination of the Milk.—A small sample of 3 to 4 cc. of the first milk withdrawn from each quarter of each cow was collected daily at the morning milking for laboratory examination. This daily examination of the fore milk was continued throughout the experiment. The laboratory tests consisted in the determination of the number of leucocytes, the pH and the chloride content, and the plating in blood agar of 1 cc. of a 1:10 dilution of the milk. As a regular procedure following the inoculation of a quarter, colonies were picked at frequent intervals from the surface of the blood agar plates and transferred to lactose broth for identification. A recent publication (9) gives routine methods employed in these tests, and reports in detail the results of daily laboratory examinations of the fore milk (from 31 quarters of 8 cows) before and after inoculation with streptococci.

Preparation of Culture for Inoculation.—A freshly prepared blood agar transplant of each strain from a human source was received from Dr. Brown. A loop of growth from this culture was inoculated into a tube of cooked meat medium and incubated for 8 hours, after which the tube was sealed with sterile mineral oil and stored in a refrigerator. In preparing the culture for inoculation, a few drops of the cooked meat medium broth were transferred to fresh bouillon and incubated overnight. A few drops of the latter culture were then transferred to fresh broth, incubated for 6½ hours, and diluted 10^{-1}.

The bovine strain which was inoculated for comparative purposes had been recovered in November, 1937, from the right hind quarter of cow M652, and this was prepared in the same manner as the human strains.

Method of Inoculation.—A glass rod approximately 2 mm. in diameter, with a smooth beaded end capable of taking up 1½ to 2 mg. of fluid, was dipped into the culture dilution and introduced into the teat just beyond the sphincter. The rod, about 110 mm. long, was drawn from a thicker piece of glass (7 mm. in diameter), thus providing it with a handle. When possible the inoculations were made in duplicate into a teat of each cow.

Cultural Characteristics and Serological Grouping of the Streptococci Inoculated.—The source and cultural characters of the streptococci are given in Table I.[1]

The human strains Nr, Fy, and T78 possessed cultural characteristics similar to the bovine culture M652, whereas the other human strains failed to ferment lactose or to alter litmus milk. Serologically all the strains were classified as belonging to group B, but some variation was found in the typing. Although culture Py failed to attack lactose, its serological typing was similar to that of the Nr and bovine strains.

RESULTS

The results of the inoculations of the udders of four cows with human and bovine double zone beta hemolytic streptococci are presented in Table II. The data show that the human cultures are

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[1] A more detailed description will be presented in a separate paper by Dr. Brown to be published in the *Journal of Bacteriology*.

capable of producing mastitis when conveyed to the udder by way of the teat canal. The three strains Nr, Fy, and T78, which are culturally similar to the bovine streptococcus, were used in seven experiments. In all these experiments the inoculations resulted in mastitis. However, the infection was limited in four. In two of the

TABLE I

The Classification of the Streptococci Employed for Inoculation Experiments

Strain No.	Serological groupings		Dextrose	Lactose	Saccharose	Maltose	Mannite	Raffinose	Inulin	Salicin	Esculin	Sorbitol	Trehalose	Methylene blue 1:5000	Litmus milk 37°C. 5 days	Sodium hippurate
	Group*	Type														
Nr†	B	III	4.4	+	+	+	–	–	–	+	–	–	+	–	+ a., c., sl.r.‡	+
Fy	B	II	4.4	+	+	+	–	–	–	+	–	–	+	–	+ a., c., sl.r.	+
T78	B	Unclassified	4.3	+	+	+	–	–	–	+	–	–	+	–	+ a., c.	+
Bovine M652	B	III	4.4	+	+	+	–	–	–	+	–	–	+	–	+ a., c., sl.r.	+
BB	B	IB	4.4	–	+	+	–	–	–	+	–	–	+	–	–	+
Py	B	III	4.4	–	+	+	–	–	–	+	–	–	+	–	–	+
Gn	B	IB	4.4	–	+	+	–	–	–	+	–	–	+	–	–	+

* Each strain was tested serologically by Dr. Lancefield for Dr. Brown.

† Nr, isolated from blood of a patient with a fatal septicemia following abortion, Mar. 23, 1936. Culture has been kept in vacuum since Apr. 15, 1936.

Fy, isolated Jan. 24, 1938, from throat of a milker with no symptoms of illness.

T78, isolated Mar. 15, 1938, from removed tonsils.

Bovine M652, isolated Nov. 30, 1937, from a blood agar plate of the fore milk from right hind quarter of cow M652.

BB, isolated from blood of a new born baby, June 2, 1930. Culture has been kept in vacuum since June 25, 1930.

Py, isolated from a catheterized specimen of urine from a woman, taken Dec. 16, 1937, from a case of cystitis.

Gn, isolated from a normal throat in 1934.

‡ a. = acid; c. = coagulated; sl.r. = slight reduction of litmus.

quarters, one inoculated with culture Nr and one with T78, the subclinical infection[2] persisted throughout the period of observation. The

[2] When subclinical mastitis or subclinical infection is referred to in this or other papers, this designation has been used simply for differentiation between the early and more advanced infections. It is realized that streptococci belonging to Minett's group I (10) are generally responsible for the more chronic form of mastitis.

TABLE II

The Results of the Inoculation of Human and Bovine Double Zone Beta Hemolytic Streptococci into the Udders of Young Cows

Cow No.	Quarter	Daily examinations of milk before inoculation*	Date of inoculation	Culture	Mastitis	Duration of mastitis (days)	Daily examinations of milk after inoculation	Number of days streptococci were present in fore milk (days)	Results
2017	LF	10	*1937* Dec. 14	Nr					
2018	LF	10	" "	"	+	34	34	34	Subclinical mastitis†
2018	LH	10	" "	"	+	24	31	21	Recovered; slight induration of quarter
2017	LH	17	" "	"	+	31	31	29	Subclinical mastitis
2018	RH	23	Dec. 21	BB	+	26	26	26	" "
2017	RF	10	Dec. 28	Py	+	8	17	3	Recovered; slight induration of quarter
2018	RF	10	Dec. 14	Bovine M652	+	34	34	34	Subclinical mastitis
2035	LF	17	" "	" "	+	31	31	31	" "
2036	LF	17	*1938* Feb. 5	Fy	+	20	57‡	9	Recovered; slight induration of quarter
2035	RH	28	" "	"	+	16	57‡	12	" "
2036	RH	28	Mar. 4	Gn	+	83	83	82	Subclinical mastitis
2035	RF	59	" "	"	+	36	60	23	Recovered; slight induration of quarter
2036	RF	59	Apr. 5	T78	+	51	51	51	Subclinical mastitis
2035	LH	39	" "	"	+	8	30	5	Recovered; slight induration of udder
2036	LH	39	Mar. 15	Bovine M652	+	72	72	72	Subclinical mastitis
			" "	" "	+	14	49	13	Recovered; slight induration of udder

* Streptococci were never detected in the milk.
† See footnote 2.
‡ Daily examinations discontinued, quarters used for other purposes.

data presented in Chart 1 include the results of the inoculation of three separate quarters with strains Nr, Fy, and T78. In one quarter inoculated with strain BB and in one inoculated

CHART 1

CHART 2

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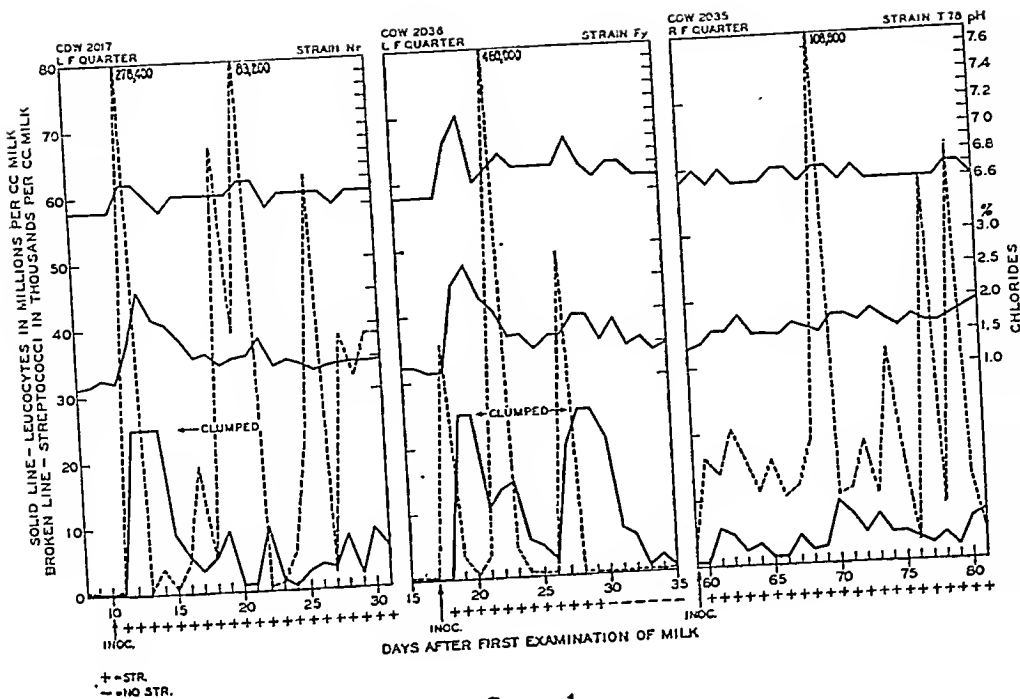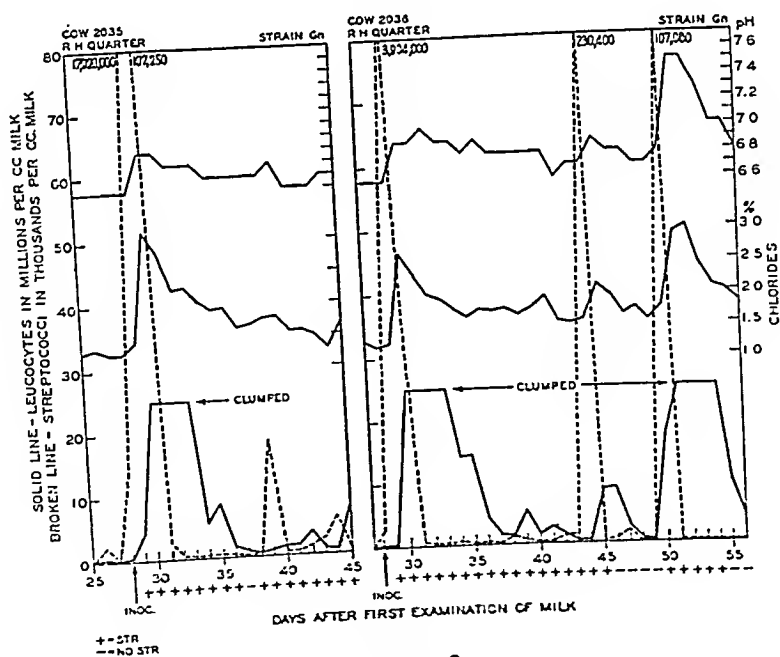
with culture Gn, the infections terminated in typical subclinical mastitis, whereas in two separate quarters inoculated with strains Py and Gn, the streptococci were not observed in the milk after 3 and 26 days respectively. Chart 2 portrays the results of the daily examination of the fore milk following the inoculation of the right hind quarters of cows 2035 and 2036 with culture Gn.

In two or three instances, streptococci were identified in the samples of middle milk or strippings when the fore milk was negative. Typical organisms sometimes reappeared in the fore milk in rather large numbers, associated with a leucocytosis and an increase in the chlorine content and alkalinity. Usually their reappearance in the milk was followed by their complete elimination. The record (Chart 2) of the right hind quarter of cow 2036, which was inoculated with the Gn strain, illustrates this point.

DISCUSSION

The human strains of double zone beta hemolytic streptococci, when inoculated into the udders of four young cows, were capable of inducing mastitis. Although not all the cultures inoculated became permanently established in the gland, the positive infections were significant. (It appears from the results of the inoculations that cow 2036 was naturally less susceptible to streptococci than the other three.)

In comparing the reaction of the udders of young cows inoculated with human double zone beta hemolytic streptococci with the results reported previously (7, 8) of the inoculation of 31 quarters of 8 young cows with the bovine strain, the most noticeable difference is that, in over half of the infections, the human organisms disappeared from the udder during the course of the experiment, while the bovine streptococci usually persisted.

The accumulated data (6, 7) warrant the suggestion that the initial inoculation of bovine or human streptococci into a quarter in no way influences the reaction of the other quarters to subsequent inoculations, provided sufficient time has elapsed between the injections. Furthermore, the milking procedure may be properly controlled by the methods developed so that the opportunity for cross-infection is eliminated.

SUMMARY

Human strains of double zone beta hemolytic streptococci produced mastitis in 11 quarters of 4 young cows. Following the acute phase of the infection, the streptococci were eliminated from 6 quarters at various intervals. However, sufficient positive infections were maintained throughout the period of observation to show that the human strains can produce the same degree of infection as bovine double zone beta hemolytic streptococci.

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QUANTITATIVE STUDIES ON ANTIBODY PURIFICATION

III. The Reaction of Dissociated Antibody with Specific Polysaccharide, and the Effect of Formaldehyde[*]

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It was shown in the first papers of this series (1, 2) that dissociation of washed pneumococcus specific precipitates and agglutinated pneumococci with strong salt solutions afforded a means, anticipated on theoretical grounds, of rapid passage from unconcentrated antipneumococcus sera to antibody solutions of great purity. Solutions were frequently obtained in which 95 to 98 per cent of the nitrogen content was precipitable by the homologous pneumococcus specific polysaccharide,[1] and in one instance an analytically pure antibody was recovered. This has also been accomplished by Goodner and Horsfall, using a modification of our method (3).

While much has been learned of the physical properties of the recovered antibody protein (4–7) its chemical reactivity with varying amounts of S has not been quantitatively charted. This was considered of interest, for it could not be foreseen whether the dissociated antibody, which represents only a variable portion[2] of the antibodies of greatly differing reactivity present in whole sera, would react as a cross section of these antibodies, or whether the dissociation process

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[1] Subsequently designated S, with the appropriate type numeral.

[2] Two Type II antipneumococcus horse sera studied recently have given very poor yields of antibody both by the salt and barium hydroxide-barium chloride methods.

was selective, removing only a portion of the antibodies different in character and reactivity from the undissociated fraction.

Experiments to gain information on this question are reported in the present paper. The reactivity of purified and native pneumococcus anticarbohydrate with formaldehyde is also considered, owing to experiments which were given considerable theoretical significance in a recent communication by Eagle (8).

EXPERIMENTAL

1. Determination of Equations.—The materials used and the methods of procedure were those referred to and described in references 1 and 2. Ratios of the components in the specific precipitates up to the equivalence zone were determined as in (9–11), and were plotted similarly, using the method of least squares when more than one line could be drawn.

In Table I are given data on the precipitin reaction between S I and (*a*) Type I antipneumococcus rabbit serum 4.75_1, (*b*) an antibody solution, 4.75_1 A, prepared from the same serum by addition of Type I pneumococci and dissociation of the washed agglutinated cells with 10 per cent NaCl solution (2), and (*c*) an antibody solution, 4.75_1 B, prepared from the salt-treated, undissociated residue of agglutinated pneumococci by elution in the cold with $Ba(OH)_2$, addition of $BaCl_2$, and neutralization (2).

Table II contains data on a Type II antipneumococcus horse serum and an antibody solution obtained from it by the method used for 4.75_1 B, above.

In Table III are given data on the precipitin reaction between S III and (*a*) Type III antipneumococcus horse serum, (*b*) a pool of three solutions, 792 L, N, P, obtained by salt dissociation of washed specific precipitates from this serum, (*c*) a single salt-dissociated antibody solution, 792 T, and (*d*) a solution, 792 Q, obtained by barium hydroxide treatment of the undissociated residues which had yielded 792 N and P.

2. Reaction of Antipneumococcus Sera and Purified Antibody with Formalin.—From the precipitin data given by Eagle (8) it is obvious that the antisera used by him were exceedingly weak. In the present experiments two Type I antipneumococcus sera were used; serum 884, preserved with phenol and merthiolate, containing 0.41 mg. of antibody N per ml., and serum 902, with no added preservative, containing 3.14 mg. of antibody N per ml.[3] The formalin used[4] was diluted 1:10 with saline. At this dilution the pH, measured with the glass electrode, was 6.40, and it was accordingly adjusted to pH 7.56 with dilute sodium hydroxide

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[3] We are indebted to Dr. R. H. Muckenfuss, Miss A. Walter, and Dr. E. M. Schryver of the New York City Department of Health for these sera.

[4] Merck's reagent "neutral" formalin.

in order to bring it near the pH of sera 884 and 902, which were 7.68 and 7.65, respectively.

In order not to deviate more than seemed advisable from the conditions used by Eagle, 12.8 ml. portions of serum 884 were allowed to stand at room temperature for one day with: A, 0.25 ml., B, 0.5 ml., and C, 4 ml. of the neutralized 1:10

TABLE I

Precipitation of Type I Antipneumococcus Rabbit Serum and Antibody Solutions by S I

Amount of S I, preparation 123,4, used	Antibody nitrogen precipitated*	Ratio antibody N to S I used	Tests on supernatants
mg.	*mg.*		
1.5 ml. serum 4.75₁, C-absorbed, diluted 3.5 to 20, 0°C.			
0.048(6)	0.290	6.0	
0.097	0.513	5.3	Excess A
0.122	0.598	4.9	" "
0.162	0.716	4.4	Trace A, trace S
0.243	0.783		Excess S
mg. antibody N pptd. = 6.7 S − 14.6 S², or 6.7 S − 11.2 S², calculated to 1 mg. N			
1.0 ml. solution 4.75₁ A, 0°C.			
0.032(4)†	0.186†	5.7	
0.048(6)	0.256	5.3	Excess A
0.097	0.419	4.3	" "
0.2	0.675		
mg. antibody N pptd. = 6.4 S − 21.6 S², or 6.4 S − 10.2 S², calculated to 1 mg. N			
1.0 ml. solution 4.75₁ B, 1:1, 0°C.			
0.048(6)	0.292	6.0	
0.097	0.507	5.2	
0.122	0.606	5.0	
0.3	0.850		
mg. antibody N pptd. = 6.7 S − 14.6 S², or 6.7 S − 11.2 S², calculated to 1 mg. N			

* Total N − S I N.

† Run on 1.5 ml. portions, with 0.048(6) mg. S I.

formalin dilution, all of these being in the range in which both specific precipitation and agglutination were inhibited in Eagle's experiments. Similarly, 6.4 ml. of serum 902 were set up with: D, 0.125 ml., E, 0.25 ml., and F, 2 ml. of the formalin dilution. Next day B showed a pH of 7.44, requiring 0.25 ml. of 0.1 N KOH (× 1.037) for adjustment to pH 7.72; C was slightly turbid, pH 6.59, requiring 1.7 ml. of the KOH solution for adjustment to pH 7.59. Solution F, presumably on account of the high globulin and high antibody content, had set to a gel (*cf.* (12)

for a discussion of the formol gel reaction); E was very viscous and faintly turbid, pH 7.33, changing to 7.66 on addition of 0.15 ml. of the KOH solution. On standing overnight in the cold a gel was formed, and this did not disintegrate at room temperature. During a period of 2.5 months in the cold A remained fluid, C set to a loose gel, and D to a firm one. The addition of 0.5 per cent of phenol to serum 902 did not prevent gel formation.

The number of amino groups combined, calculated roughly according to (8), was 2.5 for B, 3 for E, and 18 for C (assuming that 0.187 ml. N KOH would have

TABLE II

Precipitation of Type II Antipneumococcus Horse Serum and Antibody Solution by S II

Amount of S II, preparation 83B, used	Antibody nitrogen precipitated	Ratio antibody N to S II used	Tests on supernatants
mg.	mg.		
2.0 ml. serum 930, C-absorbed, 1:1, 37°C.			
0.024(6)	0.298	12.1	
0.049	0.490	10.0	
0.074	0.630	8.5	Excess A
0.099	0.724	7.3*	" "
0.197	0.784		No A or S
			Excess S

mg. antibody N pptd. = 14.0 S − 77.5 S², or 14.0 S − 49 S², calculated to 1 mg. N

2.0 ml. solution 930 B, obtained by Ba(OH)₂ dissociation after removal of 930 A by NaCl dissociation; total N, 0.417; N pptd. by S II at 37°, 0.356 mg. per ml. Experiments run at 37°

Amount of S II, preparation 83B, used	Antibody nitrogen precipitated	Ratio antibody N to S II used	Tests on supernatants
0.024(6)	0.330	13.4	
0.049	0.506†	10.3	
0.074	0.630	8.5	Excess A
			" ", trace S(?)

mg. antibody N pptd. = 15.8 S − 104 S², or 15.8 S − 62 S², calculated to 1 mg. N

* Not used in calculating equation.
† One determination only.

brought C back fully to pH 7.68), as against 21, 21, and 41 given in (8) for the same proportions of serum and formalin. As Eagle did not mention having adjusted the "neutral" formalin to a pH close to that of the serum, the discrepancy would be easily accounted for if this precaution had not been taken.

Aliquot portions of A, B, C, and D, with the usual controls, were set up at 0°C. with the amount of S I used for the original sera. A became slightly turbid, but only D precipitated rapidly. After 16 days in the cold the tubes were centrifuged for some hours at 2500 R.P.M. B and C showed only traces of precipitate and were discarded. In A, however, 0.22 mg. per ml. of antibody N had pre-

cipitated, or 54 per cent of the total, while D gave 3.36 mg., or slightly more than found originally. In the latter instance the precipitate was more pasty than that from untreated serum.

TABLE III

Precipitation of Type III Antipneumococcus Horse Serum and Antibody Solutions by S III

Amount of S III, preparation 108, used	Antibody nitrogen precipitated	Ratio antibody N to S III used	Tests on supernatants
mg.	*mg.*		
1.5 ml. serum 792, C-absorbed, 0°C.			
0.020	0.342	17.1	
0.030	0.458	15.3	
0.050	0.568	11.4	Excess A
0.075	0.570	7.6*	No A or S
0.100	0.594		Excess S

mg. antibody N pptd. = 21 S − 192 S^2, or 21 S − 110 S^2, calculated to 1 mg. N

3.0 ml. combined solutions 792 L, N, P, 0°C.			
0.023(6)	0.409	17.3	
0.039(3)	0.505	12.9	Excess A
0.050	0.539	10.8	" "
0.075	0.557	(7.4)	Doubtful†
0.100	0.563		

mg. antibody N pptd. = 22.8 S − 247 S^2, or 22.8 S − 130 S^2, calculated to 1 mg. N

2.5 ml. solution 792 T, 0°C.			
0.017(7)	0.320	18.1	
0.023(6)	0.388	16.4	Excess A
0.035(4)	0.442	12.5	" ", trace S(?)
0.059	0.478	(8.2)	No A, trace S

mg. antibody N pptd. = 24 S − 326 S^2, or 24 S − 144 S^2, calculated to 1 mg. N

2.0 ml. solution 792 Q (Ba(OH)$_2$ extracted), 0°C.			
0.020	0.490	24.5	Excess A
0.030	0.550	18.3	No A or S
0.050	0.568	11.4	" " " "
0.075	0.576		Excess S

mg. antibody N pptd. = 32.2 S − 421 S^2, or 32.2 S − 259 S^2, calculated to 1 mg. N

* Not used in calculating equation.

† Test for S with supernatant from first tube, negative; test with Felton solution from serum 792, +.

Eagle has stated (8) that the activity of purified pneumococcus antibody is not affected by formaldehyde. Since this is contrary to

Chow and Goebel (13), their experiment was repeated with the same relative amount of formaldehyde, but with a far more dilute antibody solution.

To 8 ml. of solution 701, 2 B (2), containing 0.305 mg. of antibody N and 0.314 mg. of total N per ml., or 15.8 mg. of total protein, were added at 0°C. 12.6 mg. of sodium bicarbonate and 0.75 ml. of the neutralized 1:10 formalin solution described above. The solution was allowed to stand at 0°. A test portion removed after one-half hour, the time allowed by Chow and Goebel for a solution of 20-fold concentration, showed no obvious diminution of precipitation with S I, but precipitation appeared to take place more slowly after 2.5 to 4.5 hours. The precipitates in the test portions had not dissolved next morning, but the main portion of the solution, at pH 8.09, gave only a faint turbidity with S I. Chow and Goebel showed that the reaction was reversible in acid solution, as reactivity of the antibody was restored at pH 4 at 0° for several days. A portion of our solution was precipitated with a drop of 50 per cent acetic acid and neutralized with sodium hydroxide, after which it precipitated S I. The remainder behaved similarly when acidified to litmus with a drop of N acetic acid and allowed to stand for 2 hours at room temperature. Purified pneumococcus anticarbohydrate therefore reacts with formaldehyde at alkaline reactions, with reversible loss of precipitating power, as stated by Chow and Goebel. Eagle's failure to observe this is easily explained if he failed to adjust the pH of the neutral formalin, as in the discrepancy noted above, or to add bicarbonate to the purified antibody. The difference in behavior from the antibody in whole serum would seem to be that in whole serum the non-antibody protein acts as an alkaline buffer. This is indicated by the rapid loss of precipitating power on addition of serum to unbuffered purified antibody solution containing formalin.

DISCUSSION

The data given in the tables show that purified antibody solutions prepared by dissociation of specific precipitates from antipneumococcus rabbit and horse sera follow equations of the same type as do the unconcentrated sera in their reactions with homologous type specific polysaccharide (10, 11). In many instances the equations for whole serum and the portion of its anticarbohydrate recovered either by the salt or barium method are almost identical. This is made especially clear by recalculation of the equations for the sera and antibody solutions to 1.0 mg. of antibody N for purposes of comparison. The equations then become, for rabbit serum 4.75₁, mg. antibody N precipitated = 6.7 S − 11.2 S²; and for solution 4.75₁ A, 6.4 S − 10.2 S²; and for solution 4.75₁ B, 6.7 S − 11.2 S². For Type II antipneumococcus horse serum 930 the equation becomes

14.0 S − 49 S², while for 930 B it is 15.8 S − 62 S². As may be noted from Table II, however, the differences in the latter equation are due to the higher ratio of the first and least certain point on the curve. The other points fall on the curve of the equation for serum 930. In the Type III horse serum series (Table III) the equations are 21.0 S − 110 S² for antibody N precipitated from serum 792; 22.8 S − 130 S² for that precipitated from solution 792 LNP (the three points are very close to the curve for the whole serum); 24.0 S − 144 S² for solution 792 T; and 32.2 S − 259 S² for solution Q. Only in the last instance is there evidence of a much altered combining capacity on the part of the antibody, and it is possible that solution Q was exposed to a somewhat larger excess of barium hydroxide than was ordinarily used. On the whole, then, the salt dissociation method leads to the recovery of an antibody mixture with much the same reactivities as those of the original serum, and this is also the case with the barium hydroxide-barium chloride method in favorable instances.

There appears to be a definite tendency for the values of R, the antibody N to S ratio, to be higher in the antibody solutions recovered from horse serum than in the sera themselves. The reason for this is not yet known, but aside from this evidence of slight alteration it is clear that dissociation of the specific precipitate ordinarily yields a portion of the antibody representing a cross section of the antibodies present in the serum, rather than a fraction of these of definitely higher or lower combining ratio with S. It would therefore appear that the dissociated antibody, although isolated in a state approaching and even equalling analytical purity, is still a mixture of antibodies of different reactivities. An instance of definite fractionation was, however, noted in (2), in that the antibody precipitated from a Type VIII antipneumococcus horse serum by S III was shown to be quantitatively precipitable by either S III or S VIII.

It is also shown in the experimental part that purified antibody loses reversibly its ability to precipitate polysaccharide when treated with formaldehyde at sufficiently alkaline reaction, as found by Chow and Goebel (13). A possible explanation is also given of Eagle's failure to note this (8), and of the discrepancies between our experiments and his on the action of formaldehyde on whole antipneumo-

coccus sera. In our experiments it is shown that an amount of formalin sufficient to inhibit completely specific precipitation and agglutination in the weak sera used by Eagle failed even to diminish the quantity of nitrogen precipitated by S I from an antipneumococcus horse serum containing 3.14 mg. of antibody N per ml. The same quantity of nitrogen precipitated by S I from an antipneumococcus proportion of formaldehyde, however, reduced the amount of N precipitated from a serum containing only 0.41 mg. of antibody N per ml. by one-half, and larger amounts inhibited precipitation completely.

The inhibition of specific precipitation by formaldehyde has been cited by Eagle (8) in favor of his restatement of the Bordet hypothesis of specific precipitation and against the qualitative (14) and quantitative (10) restatements of the Ehrlich hypothesis made by Marrack and by one of us and Kendall. It is, however, a difficult matter to devise experiments permitting a decision between two hypotheses which differ in as few essentials as do the two in question when translated into modern terms. For this reason Eagle's results, in so far as they are at all pertinent, seem entirely consistent with the chemical theory of specific precipitation. For example, if antigen-antibody precipitation consists in the building up of large aggregates by union of multivalent antigen with multivalent antibody (a view adopted only recently by Eagle, as well (15)) alteration of certain reactive groupings on the antibody by combination with formaldehyde might prevent their participation in precipitate formation. With fewer reactive groupings available on the antibody, the range of proportions in which precipitation would occur would become restricted. It would certainly appear reasonable to interpret the toxin-antitoxin flocculation data (Table I) in Eagle's paper in this way, especially as the number of different flocculation times diminishes progressively as the amount of formaldehyde in the antitoxin is increased. This interpretation is also consistent with the newer data on the combining proportions and their mechanism of formation recorded by Pappenheimer and Robinson (16). A similar restriction in combining proportions would be expected in the S-anti-S reaction. Partial precipitation would occur when enough formaldehyde had been added to prevent the antibodies with fewest combining groups from building up large aggregates. Complete inhibition of precipitation could

easily occur with still larger amounts, although combination of S and anti-S might still take place with formation of soluble compounds of smaller molecular size. It would appear, therefore, that the results in question may be interpreted according to inclination. The observation that pneumococcus anticarbohydrate produced by the horse is not precipitated on dilution with water after treatment with formalin (8) also appears irrelevant, as antipneumococcus rabbit sera are seldom precipitated by water (17) and still yield specific precipitates without difficulty. The antibody involved in the toxin-antitoxin flocculation is also water-soluble.

The immunological behavior of antibody recovered from antipneumococcus horse serum according to (1) is considered "remarkable" by Marrack and Duff (18) in its failure to yield much precipitate with an antiserum to whole globulin and in the quantitative aspects of its precipitation of an antiserum to specific precipitate. This' behavior, however, would seem precisely that expected from the ultracentrifugal studies reported in (4), in which it was shown that pneumococcus anticarbohydrate in the horse sediments at a rate corresponding to a fraction of high molecular weight present only in small quantities in normal horse serum. Pneumococcus anticarbohydrate recovered by the dissociation procedure should therefore react with only a small proportion of the antibodies in antisera to normal horse serum globulin, but should precipitate most of the antibodies in an antiserum to specific precipitate if added in amounts commensurate with its molecular size (19). This behavior is reported by Marrack and Duff.

SUMMARY

1. Tested quantitatively, antibody recovered by dissociation of specific precipitates from antipneumococcus sera reacts with homologous polysaccharide almost as does the antibody in the original sera.

2. The dissociation methods employed appear to yield a portion of all of the anticarbohydrates of differing reactivities in the sera, rather than a fraction of low or high reactivity.

3. The reversible inactivation of purified pneumococcus anticarbohydrate by formaldehyde is confirmed, and the failure of data on the

formaldehyde-antibody reaction to permit a choice between alternative theories of specific precipitation is shown.

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NUTRITIONAL CYTOPENIA (VITAMIN M DEFICIENCY) IN THE MONKEY*

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PLATE 50

In 1935 it was reported from this laboratory that monkeys given a diet deficient in some factor of the vitamin B complex developed an anemia, leukopenia, and in many cases gingivitis and diarrhea, and that all animals receiving such a diet died from the deficiency (1). The syndrome was prevented by supplementing the deficient diet with yeast or a liver extract (2). We have recently reported the failure of nicotinic acid to prevent this nutritional cytopenia (3). It is the purpose of this paper to describe the manifestations of this deficiency, and to present evidence showing that the syndrome is not a result of a deficiency of any of the chemically known vitamins. We have proposed the designation vitamin M for the substance in yeast and liver which prevents nutritional cytopenia in the monkey (3).

Anemias have been produced experimentally in a number of species by dietary deficiency. Wills and Bilimoria (4), Wills and Stewart (5), and Johnstone and Reed (6) have produced anemias in monkeys by dietary means. Rhoads and Miller (7, 8) have reported the irregular appearance of an anemia in dogs fed a diet productive of chronic black tongue and "apparently lacking a substance closely associated with vitamin B_2G." Spies and Dowling (9) have also noted an anemia

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in dogs on a black tongue-producing diet. Miller and Rhoads (8, 10) have further reported that a certain number of the dogs placed on their black tongue-producing diet developed "an ulcerative stomatitis associated with leucopenia and granulopenia." György et al. (11) have produced a somewhat similar syndrome in rats on a diet deficient in some component of the vitamin B complex. More recently he has reported (12) that nicotinic acid prevented this nutritional disturbance. Hogan et al. (13) have reported the development of a nutritional anemia in the pigeon and have stated that this condition did not yield to treatment with riboflavin, vitamin B, or an antidermatitis (rat) concentrate, but did respond to the administration of certain curative agents. Fouts et al. (14, 15) reported that a rice polish extract rich in vitamin B_6 supplemented a purified diet and prevented the appearance of a severe microcytic hypochromic anemia and death in puppies. Gall (16) and Miller and Rhoads (17) reported that guinea pigs seem to require some one or more substances in the diet for normal hematopoiesis. Kyer and Bethell (18) reported that a nutritional anemia in the rat might be prevented by the administration of a vitamin B_4 concentrate.

EXPERIMENTAL

Young immature monkeys (*Macaca mulatta*) weighing approximately 2 kilos were purchased from animal dealers. Upon arrival they were given a mixed diet consisting of grains, bread, fresh vegetables and fruits, and dried dog food. During a preliminary period of observation of at least 3 weeks, any animals that showed diarrhea, cough, anorexia, or other symptoms which did not abate shortly were discarded. Those animals which appeared to be normal were then separated, weighed, and bled. After this preliminary period of observation at least two blood counts were taken on each monkey before it was placed on an experimental diet. Some of the animals were in our laboratory more than a year before they were started on experiment; during this period each gained approximately 1 kilo in weight.

After an animal was found to be normal it was started on the experimental diet and thereafter was weighed and examined, and blood was drawn for study at weekly intervals, or oftener as the nature of the experiment or condition of the animal required. Some animals were bled daily near death or after the administration of a supplement to the deficient diet. The monkeys were kept in large metal metabolism cages with wire mesh bottoms. No effort was made to prevent coprophagy. The monkeys were housed in a light, airy room, but no attempt was made to expose them to direct sunlight. The temperature of the room was maintained as nearly constant as was possible; the room was heated continuously in winter, and the temperature rarely fell below 20°C. Clean tap water was kept in the cages at all times. The animals were fed once a day, usually about noon, but never before blood was drawn on that particular day. Weighings were made by placing the monkey in a tared cage on a direct reading balance. The techniques for blood determinations were the same as those described elsewhere (19).

Normal animals on a mixed diet were usually kept in the same room simultaneously with the experimental animals. In none of these have we ever observed a symptom complex resembling in any way that produced in our experimental animals. That the hygienic conditions in our quarters are satisfactory for the monkey is evident from the fact that we have kept certain animals in our laboratory for more than 2 years, and they have remained in good health and developed normally.

The experimental diet (No. 600) has been altered only slightly from that previously reported (1). In recent years we have used somewhat larger monkeys and hence felt it advisable to increase the caloric intake. Vitamin C is now supplied in the form of tablets of ascorbic acid (Merck), and in our more recent experiments a more complete salt-mixture has been used (20). The quantities fed each monkey per day were as follows:

	gm.
Casein, washed with dilute alcohol[1]	10
Rice, polished, uncoated	50
Ground whole wheat	15
Salt mixture, Hubbell, Mendel, and Wakeman (20)	3
Sodium chloride	2
Cod liver oil	3
Ascorbic acid (Merck)	0.01

The casein, rice, salt mixture, and salt were mixed in an enameled boiler and tap water was added. This mixture was cooked on a steam bath with frequent stirring for 1½ hours. The whole wheat was then added and the cooking continued for ½ hour longer. The diet was then cooled in front of a fan, and the cod liver oil was mixed with the cooled material. The diet was cooked fresh every second day and was stored in a refrigerator until used. The ascorbic acid was fed separately in the form of tablets which the monkeys ate readily. Thiamin chloride was fed to one monkey in the same way. The monkey which was given extralin received 4 pulvules of this preparation before its basal diet, and consumed them readily. All other orally administered supplements were mixed with the diet just before feeding.

The diet, as it was given to the monkeys, had the consistency of a thick paste or mush, and was fed in earthenware dishes. Each animal was offered the amount of diet indicated above and in nearly every case the entire portion was eaten. In a few animals it was necessary to develop a taste for the diet by mixing a small amount of fresh banana with the portion for the first 3 or 4 days, after which they readily ate the diet without the banana. When a supplement was added and the monkey did not eat all of its diet, the amount of basal diet was decreased somewhat to make certain that all of the supplement was consumed.

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[1] Casein, edible, muriatic, manufactured by the Casein Manufacturing Company of America, New York; washed with 60 per cent alcohol in this laboratory by the method of Sherman and Spohn (21).

Monkey 28 received the following diet, which is a modification of the Goldberger black tongue-producing diet No. 268 (22).

	gm.
Corn meal, white.	40
Cowpeas, black eyed, ground.	5
Casein, washed with dilute alcohol.	10
Cottonseed oil.	3
Cod liver oil.	3
Salt mixture, Osborne and Mendel (23).	3
Sodium chloride.	2
Ascorbic acid.	0.01

The ingredients, except for the cottonseed oil, cod liver oil, and ascorbic acid, were cooked in an enameled boiler for 2 hours. After cooling, the cottonseed oil and cod liver oil were added. The ascorbic acid was fed separately. The monkey was cared for in the same way as were animals on the other diet.

RESULTS AND DISCUSSION

Since space does not permit the presentation of complete hematological data on all of the animals, we are giving in Table I the data on individual animals at the most critical points in the experiments. Hematological data on normal monkeys under the conditions existing in this laboratory have been presented elsewhere (19), but for ready reference the average normal figures and the normal ranges for blood elements are repeated at the beginning of Table I. The text-figures give somewhat more complete results on 5 of the monkeys. In the following paragraphs the data are discussed.

Deficient Diet.—Monkeys 13, 20, 22, 25, 26, and 30 received the deficient diet alone (diet 600). In every case they showed the symptom complex previously described: leukopenia, neutropenia, anemia, and loss of weight. A decrease in packed cell volume (hematocrit) was also seen. There also appeared to be a decrease in the number of platelets, although the data for this blood element are more meager. The dramatic increase in number of platelets to 1,555,000 per c.mm. following the administration of yeast to monkey 27 (Text-fig. 4) would also suggest that a thrombocytopenia is a part of the picture of this nutritional deficiency. No consistent variation in the clotting time has been observed. Ulceration of the gums and diarrhea were less consistently seen than was the cytopenia. The gum ulceration, as would be expected, appeared to be more closely correlated with

TABLE I

Hematological Data on 22 Monkeys at the Most Significant Points in the Experiments. The Average Normal and Normal Range for Blood Elements in Monkeys of This Colony Are Given for Comparison

Monkey No.	Time on diet	W.B.C. per c.mm.	Lymphocytes per c.mm.	Neutrophils per c.mm.	Platelets per c.mm.	R.B.C. per c.mm.	Reticulocytes	Hemoglobin	Hematocrit	Weight	Diet and remarks
	days	thousands	thousands	thousands	thousands	millions	per cent	per cent	per cent	gm.	
19 normal mon- keys		15.1 9.7– 20.5	8.8 5.0– 13.0	5.7 2.0– 9.0	475 318– 632	5.2 4.6– 5.8	0.6 0.56– 0.64	12.2 10.9– 13.5	40.0 36.4– 43.6		Average normal Normal range
13 ♀	3	9.3	5.9	2.9		4.65		11.9		2037	Deficient diet 600*
	64	1.4	1.0	0.4		1.93		5.2		1330	Died
20 ♀	0	10.0	7.0	2.1	234	5.38	0.9	11.8	39.0	3860	Deficient diet 600*
	54	1.1	1.1	0.1	175	3.24	0.6	8.2	26.2	3400	
	55	2.8	2.7	0.6	76	3.48	0.3	8.2	27.5		Died
22 ♂	−2	7.9	3.0	4.2		6.56		12.5		2700	Deficient diet 600*
	61	3.7	3.5	0.1		0.95		2.2	7.2	2405	Died 62nd day
25 ♀	1	16.0	7.8	7.7		4.82		9.9		2215	Deficient diet 600*
	25	0.7	0.5	0.2		2.84		6.6		1715	Died 26th day
26 ♂	1	20.7	8.7	11.2		5.16		12.1		2455	Diet 600*
	27	5.4	3.5	1.6		4.39		12.6			
	42	8.4	4.2	4.0		2.74		6.4	22.8	2060	Died 44th day; photo- graphed
30 ♂	0	17.4	13.4	3.4	725	5.00	1.2	11.1	43.5	2490	Diet 600†
	59	4.7	2.8	1.8	635	3.30	0.3	7.7	26.0	2110	Gums ulcerated; appetite good
	94	0.5				1.40		3.8	11.0	1445	Died
28 ♂	−3	22.8	7.5	14.4	412	5.02	0.8	12.3	41.5	2790	Modified Goldberger diet
	72	6.7	2.0	4.2	463	4.27	0.2	10.8	30.7	2502	Gums ulcerated
	114	4.1	2.4	1.6	635	2.08	0.9	6.5	18.8	2532	Diarrhea
	162	1.2			278	1.77	0.4	5.0	13.0	1719	Died 163rd day
23 ♀	−2	32.0	4.5	25.9		4.63		13.6		2510	Diet 600*
	45	5.4	2.5	2.5		4.21		9.7	32.0	2145	Gums ulcerated. 6 gm. yeast added to diet‡
	53	7.6	1.2	5.4		2.66		9.2	27.2		Died (heart blood)

* Received the original diet (1) which contained Osborne and Mendel salt mixture (23), and 4 gm. of orange daily as a source of vitamin C.

† Received 0.01 gm. of ascorbic acid daily in place of the orange.

‡ Dried brewers' yeast, vita-food, red label, supplied by the Vitamin Food Company, New York.

TABLE I—*Continued*

Monkey No.	Time on diet	W.B.C. per c.mm.	Lymphocytes per c.mm.	Neutrophils per c.mm.	Platelets per c.mm.	R.B.C. per c.mm.	Reticulocytes	Hemoglobin	Hematocrit	Weight	Diet and remarks
	days	*thousands*	*thousands*	*thousands*	*thousands*	*millions*	*per cent*	*per cent*	*per cent*	*gm.*	
24 ♀	0	16.7	9.4	6.7		5.66		11.0		2265	Diet 600* + 10 gm. yeast‡
	200	15.0	9.5	4.4	280	5.69	0.4	12.5	44.0	2849	Yeast reduced to 5 gm. daily
	429	17.8	7.46	9.59	428	6.18	0.7	12.8	45.3		Yeast reduced to 2.5 gm. daily
	664	4.1	1.35	2.54	56	1.61	4.3	5.7	18.5	2890	10 gm. yeast added daily, 5 days prior to death; died 665th day
27 ♂	1	10.6	6.3	4.2		4.6	0.6	10.4	37.0	1760	Diet 600* + 10 gm. skim milk powder daily
	34	4.8	3.5	0.9	280	3.72	0.6	7.5	25.2	1956	Milk withdrawn; 10 gm. yeast‡ added daily
	45	6.1	4.4	1.4	1555	3.9	4.0	10.0	30.3	1860	Stools normal
	498	20.4	16.5	3.7		5.78	0.6	12.3	41.0	3770	
	596	13.0	8.9	4.0	705	5.46		13.0	36.5	3427	Normal this date
29 ♂	1	13.8	5.5	8.0	1115	5.03	0.3	11.7	37.0	3016	Diet 600* + 4 pulvules extralin§
	572	11.8	3.1	8.6	380	4.08	0.7	10.7	32.3	3495	Paralysis; off diet
32 ♀	−2	9.4	7.0	1.8	374	5.27	0.1	12.4	41.0	3275	Diet 600† + 2 gm. Lilly liver extract daily
	630	12.1	7.5	3.6		5.40		11.8		3935	Diet 600‖ + 2 gm. Lilly liver extract. Menstruated
	728	21.1	12.9	6.9		5.17		10.5		4955	Liver extract withheld
	799	4.1	1.4	2.4		4.12		8.5		3300	Gums ulcerated; died 802nd day
34 ♂	0	17.1	10.4	5.3	630	5.66	0.4	14.6	40.6	3080	Diet 600† + 1 cc. Lederle liver extract weekly
	56	8.0	3.5	3.9	510	5.24	0.4	13.2	35.0	3195	
	104	1.6	0.9	0.3		2.69				1579	Died 105th day
31 ♂	0	11.9	6.3	4.9	824	5.36	0.9	13.9	42.0	2667	Diet 600† + 5 mg. Cu daily
	58	9.4	6.9	2.0	221	4.80	0.0	12.6	36.5	2695	Gums necrotic
	99	0.9	0.8	0.1	337	5.6	0.2	10.6	29.3	1817	Gums very necrotic; died 100th day
33 ♀	0	14.1	8.8	4.1	578	4.6	1.0	10.6	38.5	2340	Diet 600†
	34	9.0	4.5	4.4	415	3.16	0.0	10.6	27.5	2295	5 mg. Cu added to diet daily
	48	4.0	3.9	0.1	215	2.98	0.1	7.6	22.5	2285	
	56	3.4	2.4	0.8		0.73			4.5	2365	Died 57th day

§ Extralin is a liver-stomach preparation manufactured by Eli Lilly and Company.

‖ Received the diet of composition indicated in the text, which contained the salt mixture of Hubbell, Mendel, and Wakeman (20).

TABLE I—*Concluded*

Monkey No.	Time on diet	W.B.C. per c.mm.	Lymphocytes per c.mm.	Neutrophils per c.mm.	Platelets per c.mm.	R.B.C. per c.mm.	Reticulocytes	Hemoglobin	Hematocrit	Weight	Diet and remarks
	days	*thousands*	*thousands*	*thousands*	*thousands*	*millions*	*per cent*	*per cent*	*per cent*	*gm.*	
43 ♂	0	15.0	7.68	5.72		5.72		10.5		2200	Diet 600‖ + 10 mg. nicotinic acid daily
	71	2.2				4.38		8.21		1580	Died; gums necrotic
40 ♀	−1	17.9	10.05	6.28		4.35		11.00		3400	Diet 600‖ + 10 mg. nicotinic acid
	55	4.9	2.47	2.13		3.14		7.52		2825	Alopecia face and chest
	89	3.0	0.94	2.00		2.75	0.5	6.13		2595	Extensive alopecia face; *given Dakin-West liver concentrate***
	105	3.5	1.51	1.95		2.89	2.4	6.75		2475	
	120	2.1	1.42	0.66		2.39	0.2	5.98		2235	Died 122nd day
37 ♂	−1	8.7	6.18	2.26		5.39		10.1		3465	Diet 600‖ + 10 mg. nicotinic acid
	74	0.9	0.68	0.17		3.85		10.36		2280	Died
36 ♀	−1	8.0	4.88	2.9		4.93		10.71		3465	Diet 600‖ + 1 mg. riboflavin by mouth
	48	3.4	1.9	1.5		4.6		12.28		2695	No diarrhea; gums necrotic; died 51st day
45 ♀	−8	9.5	5.05	4.10		5.38		10.71		2055	Diet 600‖ + 50 mg. nicotinic acid + 1 mg. riboflavin parenterally
	63	2.4	1.42	0.79		3.67		8.10		1865	0.5 gm. nucleic acid daily added
	76	30.2	10.26	19.32		3.88		9.36		1630	Died 77th day
44 ♂	0	19.3	14.82	3.66		5.63		14.55		2685	Diet 600] + 10 mg. nicotinic acid + 1 mg. riboflavin parenterally
	89	3.7	1.70	1.81		3.44		7.18		1845	Died; diarrhea; gums ulcerated
42 ♀		31.7	17.76	11.40		5.53		13.33		2280	Diet 600] + 50 mg. nicotinic acid + 1 mg. riboflavin + 1 mg. thiamin
	50	5.4	1.40	3.78		4.58		8.96		1690	Diarrhea; died 57th day; gums necrotic

** Received a preparation from 50 gm. of Lilly's liver extract, prepared by the method described by Dakin, Ungley, and West (26).

the leukopenia and granulopenia than with the anemia (note monkeys 31 and 36). Unless the diet was supplemented with some active material, the animals died in about 100 days.
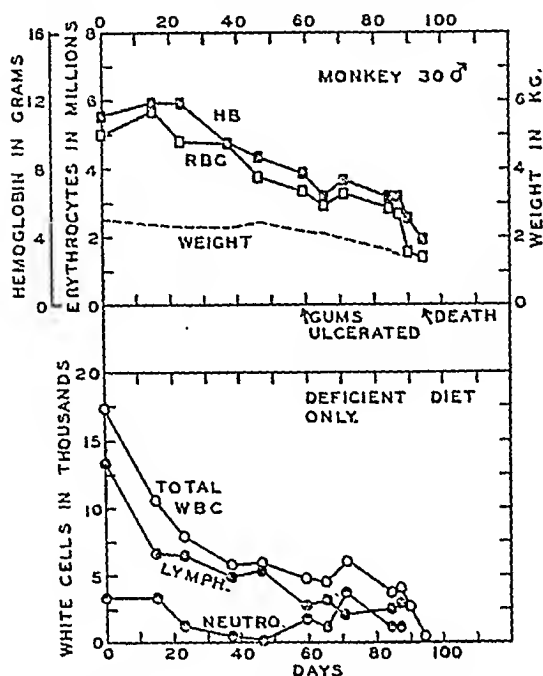
TEXT-FIG. 1. Hematological and weight data on a monkey which received the deficient diet without supplement.
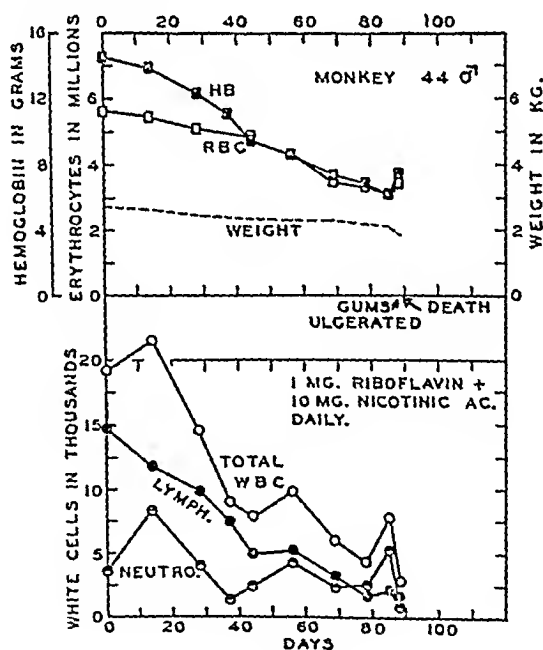
TEXT-FIG. 2. Hematological and weight data on a monkey which received the deficient diet supplemented with 1 mg. riboflavin parenterally and 10 mg. nicotinic acid by mouth each day.

The degree of anemia seemed to vary somewhat with the animal. Erythrocyte counts below 1,000,000 per c.mm. have been found; however, the counts were more frequently around 3,000,000 at or before death. The decrease in hemoglobin appeared to parallel the decrease in erythrocytes. Hence, the color index did not vary greatly from normal for the monkey. The packed cell volume also paralleled the erythrocyte numbers, so the volume index did not depart significantly from normal. A few of the animals died before the anemia had become marked.

The average total white blood cell count for the monkey (15,000 per c.mm.) is considerably above that of man. Therefore, a total white cell count of 5000 or less would indicate a definite leukopenic state. Several of the monkeys on the
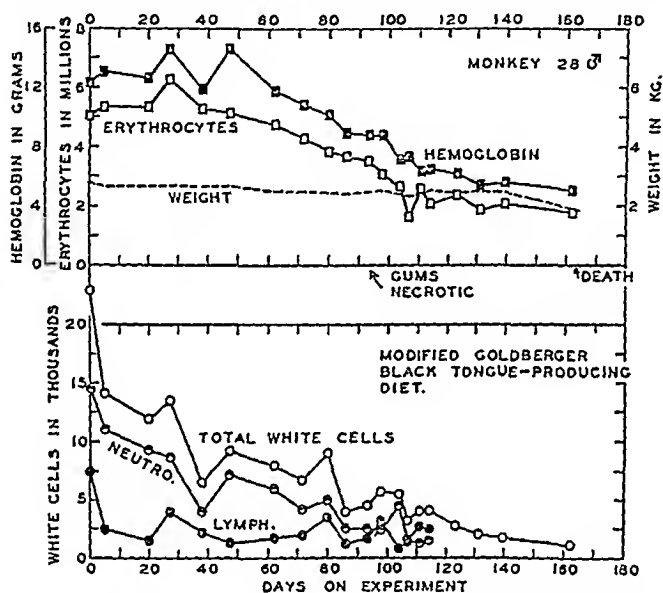
TEXT-FIG. 3. Blood changes in a monkey which received a modification of the Goldberger black tongue-producing diet.

deficient diet exhibited before death white cell counts of less than 1000 per c.mm. The most frequent finding, however, was a total white count of approximately 3000 cells per c.mm. Usually, but not always, there was a relative granulocyto-penia. It is suggestive that those animals which survived the deficiency for the longest periods also tended to maintain the normal *relative* distribution of granu-locytes and lymphocytes. That the leukopenia was not the result of the gingival infection is suggested by the fact that we have frequently seen animals die with very low white cell counts without showing gum lesions. The converse has not been true, however. Usually the leukopenia appeared to precede shortly the anemia.

Terminal rises in the numbers of all cellular elements have sometimes been
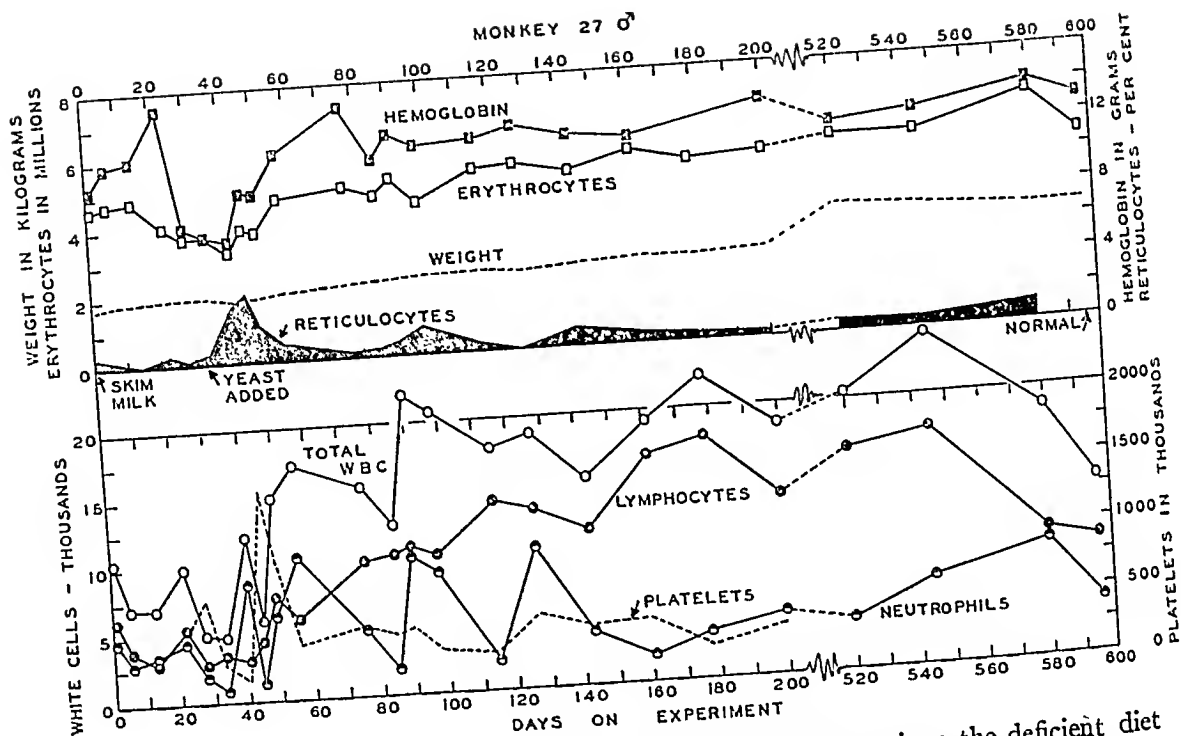
TEXT-FIG. 4. Blood picture of monkey 27. This animal was given the deficient diet supplemented with 10 gm. of skim milk powder daily. A mild anemia and distinct leukopenia had developed by the 34th day. At this point the milk powder was discontinued and replaced by 10 gm. of dried yeast daily. There was an immediate increase in reticulocytes and platelets and the entire blood picture slowly returned to normal.
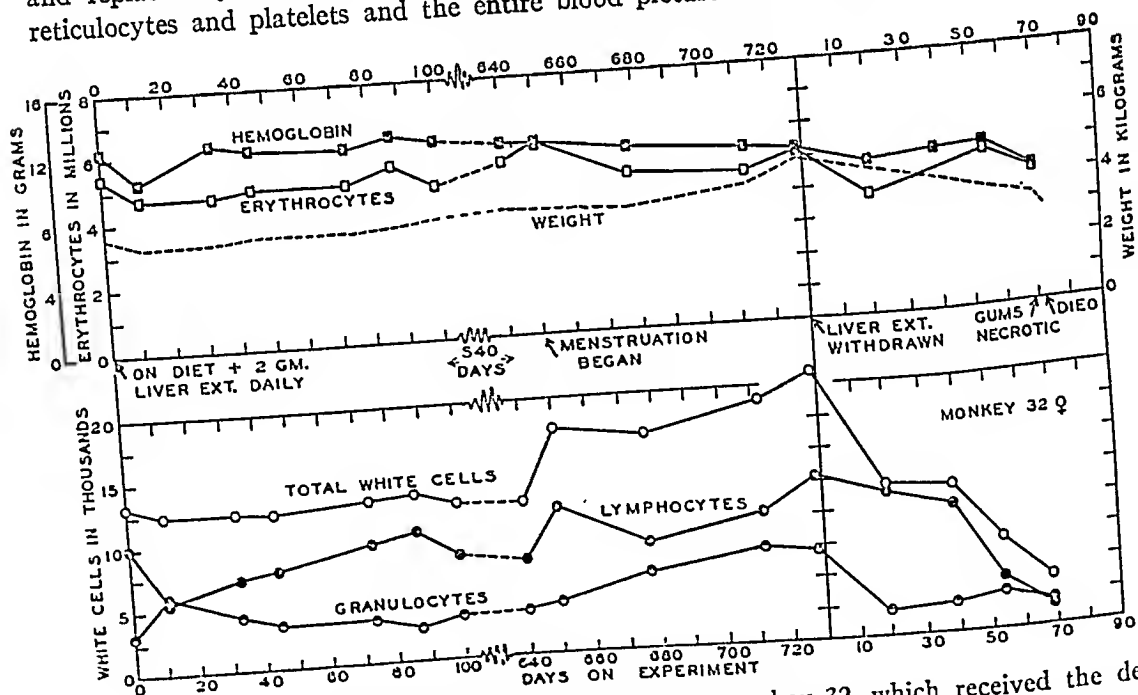
TEXT-FIG. 5. Hematological and weight data on monkey 32, which received the de ficient diet supplemented with 2 gm. of Lilly's liver extract daily. The animal gaine in weight and developed normally, and at the end of 2 years the blood picture was sti normal. At this time the liver extract was withdrawn, and the animal died in 72 day showing the typical symptoms of weight loss, leukopenia, and ulceration of the gums.

932

seen in the last two or three days of life. These rises have never increased the counts to normal values, however. They may possibly be explained by dehydration of the moribund animal.

Black Tongue-Producing Diet.—Monkey 28 was fed the modified Goldberger diet. It also developed a severe leukopenia and anemia, with marked ulceration of the gums and a diarrhea. In contrast with the results of Miller and Rhoads in dogs (7, 10) and in swine (24) there appeared to be no tendency to spontaneous remission. Likewise, we have never observed a spontaneous remission in any monkey on diet 600. Also, in contrast with the findings of Rhoads and Miller in dogs (7), we have not observed a significant consistent change in either the color index or the volume index in this deficiency in the monkey. Further study will be necessary to determine whether these differences are due to variations in the response of the several species to the same deficiency, or whether the conditions are manifestations of deficiencies of distinct nutritional factors.

Although it is logical to assume that the blood changes in monkeys on our diet 600 and on this modified black tongue-producing diet are the result of the same deficiency, this point remains to be proved. Experiments are under way to determine the effect of supplementing the black tongue-producing diet with nicotinic acid. The blood findings on the 2 monkeys reported by Johnstone and Reed (6) are strikingly similar to those which we observed.

Yeast.—Monkey 24 was given the deficient diet supplemented with 10 gm. of dried brewers' yeast (vita-food, red label). The animal gained in weight and size, and dentition proceeded normally. After 200 days the blood picture was still normal; at that time the amount of yeast was reduced to 5 gm. daily. On the 429th day the blood picture was still normal, and at that time the daily amount of yeast was reduced to 2.5 gm. Following that reduction the monkey developed a severe cytopenia and died on the 665th day, showing the following terminal values: total white cells, 4100 per c.mm.; platelets, 56,000 per c.mm.; erythrocytes, 1,610,000 per c.mm.; hemoglobin, 5.7 gm. per 100 cc.; and packed cell volume, 18.5 per cent. 5 days before death the daily dosage of yeast was increased to 10 gm. This was followed by an increase in reticulocytes to 4.3 per cent, but the increased amount of yeast did not prevent death. Although 2.5 gm. of yeast daily did not prevent the deficiency manifestations, that amount did appear to prolong life. It appears, therefore, that the minimum protective dose of this preparation of dried brewers' yeast was between 2.5 and 5 gm. daily.

Monkey 27 was started on experiment with the deficient diet supplemented with 10 gm. of skim milk powder daily (see Table I and Text-fig. 4). By the 34th day a mild cytopenia had developed. At this point the milk powder was replaced by 10 gm. of dried brewers' yeast daily. As can be seen by an inspection of Text-fig. 4, there was a sharp rise in reticulocytes to 4 per cent and a simultaneous

increase in platelets to 1,555,000 per c.mm. There was a slower return of white cells, erythrocytes, hemoglobin, and packed cell volume to normal levels. On the 596th day the blood picture was still normal.

It is evident from these two experiments that brewers' yeast contains a factor which is essential in the maintenance of proper hematopoietic function in the monkey. In sufficient quantities yeast is able to prevent nutritional cytopenia in the monkey, and must therefore be regarded as a reliable source of vitamin M.

Liver Extract.—The effectiveness of several liver preparations in the prevention of nutritional cytopenia was studied. Monkey 32 was given the deficient diet supplemented with 2 gm. of Lilly's liver extract (343 powder), which is essentially Cohn's fraction G (25). The animal gained in weight and height, and the blood picture was still normal at the end of 2 years. At the start of the experiment the monkey was juvenile, but during the experiment it passed puberty and began to menstruate. Dentition proceeded normally with the loss of deciduous teeth and the eruption of permanent teeth. At the end of the second year the liver extract was withdrawn. The animal died in 72 days following the withdrawal of the liver extract, after showing loss of weight, a marked leukopenia, and ulceration of the gums. Monkey 29 was given the diet supplemented with 4 pulvules of extralin, a liver-stomach preparation. This animal gained in weight and height, dentition proceeded normally, and the blood picture was still normal at the end of 572 days. The monkey developed a paralysis of unknown etiology during the latter part of the experiment; this paralysis will be discussed later.

Monkey 34 was given the deficient diet supplemented with 1 cc. per week of Lederle liver extract parenterally. This dosage was based upon the amount of the extract necessary to keep a pernicious anemia patient in remission. The monkey developed the severe leukopenia and anemia typical of vitamin M deficiency, and died on the 105th day. On the day before death the total white cells numbered 1600 per c.mm., and the total erythrocyte count was 2,690,000 per c.mm. It is apparent that this amount of Lederle liver solution did not protect against nutritional cytopenia. One of the animals (monkey 40) was given a concentrate from Lilly's liver extract prepared according to the method of Dakin, Ungley, and West (26) after a marked cytopenia had developed. Although this was followed by an increase in reticulocytes to 2.4 per cent, the condition of the animal became progressively worse and it died on the 122nd day.

It is evident from the experiments on monkeys 29 and 32 that certain liver extracts given orally were effective in preventing nutritional cytopenia over long periods of time, and as supplements to the deficient diet were capable of promoting normal growth and development.

The experiments with more concentrated preparations of the anti-pernicious anemia factor were less conclusive.

Riboflavin.—It seemed possible that nutritional cytopenia might be the result of a deficiency of riboflavin; consequently, the effectiveness of synthetic riboflavin was investigated. Diet 600 contains some riboflavin in the whole wheat, but rats on a similar diet containing 35 per cent whole wheat eventually developed the characteristic signs of flavin deficiency (27).

The requirement of the monkey for riboflavin is a matter of conjecture only. Estimates of the human requirement have been from 400 Bourquin-Sherman units for children to 600 units for adults (28). Accepting the tentative conversion factor of 1 unit as equivalent to between 2 and 3 micrograms of riboflavin (29, 30), the human requirement would be between 1 and 2 mg. of riboflavin daily. The requirement of a young 2 kilo monkey should be considerably less. We therefore decided to give 1 mg. of synthetic riboflavin daily to certain monkeys as supplements to the deficient diet. 4 monkeys (36, 42, 44, and 45) were given this dosage either alone or in combination with nicotinic acid, or with nicotinic acid and thiamin chloride. The riboflavin was supplied to us in sterile ampules, each containing 1 mg. riboflavin in 2 cc. of solution. Monkey 36 was given the riboflavin mixed with the basal diet. The somewhat bitter taste of the riboflavin seemed to render the diet unpalatable, however, so the method of administration was changed and the riboflavin was given subcutaneously during the latter part of the experiment. The other 3 animals received it subcutaneously throughout the experiment. Monkey 44 was also given 10 mg. of nicotinic acid daily mixed with the basal diet (Text-fig. 2). Monkey 45 received 50 mg. of nicotinic acid daily, while monkey 42 received 50 mg. of nicotinic acid and 1 mg. of thiamin chloride daily, in addition to the riboflavin.

It is evident from the data given in Table I on these 4 animals that riboflavin, either alone or in combination with nicotinic acid or with nicotinic acid and thiamin chloride, did not prolong life or prevent a marked leukopenia. It is possible that the riboflavin tended to sustain the hemoglobin and erythrocyte numbers, since monkey 36 did not develop an anemia. This would not be surprising in view of the reported value of riboflavin in hemoglobin production (31). The other 3 monkeys showed a distinct anemia, however, and the sustained erythrocyte numbers and hemoglobin in monkey 36 can probably be explained by two facts: this animal was somewhat larger (3.5 kilos) than most of our animals; and the survival period was shorter. We have observed that the anemia appears to develop more slowly than the leukopenia, and that larger monkeys die before they show more than moderate anemia.

Monkey 45 was given 0.5 gm. of yeast nucleic acid daily after marked cytopenia had developed. This was followed by a dramatic increase in leukocytes to a total of 30,000 per c.mm. The animal died on the 77th day, nevertheless. At autopsy numerous small abscesses were found throughout the wall of the intestine and in certain other abdominal viscera. In the presence of this complication it is im-

possible to evaluate the rôle of the nucleic acid. Further investigation of this substance is planned.

It is apparent that riboflavin, either alone or in combination with nicotinic acid and thiamin chloride, was ineffective in the prevention of nutritional cytopenia.

Nicotinic Acid.—In another place (3) we have reported the failure of nicotinic acid to prevent nutritional cytopenia in the monkey, and in that paper detailed hematological data were given on 2 monkeys. Table I gives the most significant results from the 6 monkeys which received nicotinic acid (monkeys 37, 40, 42, 43, 44, and 45) and Text-fig. 2 gives complete data on one of them. These data are also discussed in the paragraph on riboflavin. Although it is conceivable that nicotinic acid amide or some other pyridine derivatives might be effective, it is clear that nicotinic acid is not identical with the substance that prevents nutritional cytopenia in the monkey (vitamin M).

Copper.—Originally we used the salt mixture of Osborne and Mendel (23) as a source of inorganic elements. This mixture furnishes abundant iron, but contains copper only as it is incidentally found as a contaminant of the chemicals used. Therefore, we gave one animal (monkey 31) the deficient diet prepared with this salt mixture but supplemented with 5 mg. of copper daily as copper sulfate. It developed a severe leukopenia (Table I) and marked necrosis of the gums (Figs. 2 and 3), but did not show the severe anemia which is commonly observed. Monkey 33 was then given the deficient diet without copper supplement and allowed to develop a moderate anemia. At this point 5 mg. of copper daily was added to the diet. There was no increase in erythrocytes and no reticulocyte response, and the animal showed an erythrocyte count of 730,000 just before death.

Since October, 1937, all the animals used have received 3 gm. daily of the improved salt mixture of Hubbell, Mendel, and Wakeman (20), and hence have received more than 1 mg. of copper daily from this source alone. Moderately severe anemia has appeared in approximately the same percentage of comparable animals as was observed before the change in salt mixture, and one monkey has exhibited a severe anemia since the change. No appreciable alteration of the total white or differential counts has been observed since the change of salt mixture and the consequent increase in copper content of the diet.

Although the apparent stimulating action on erythropoiesis of moderately large doses of copper sulfate in the one animal requires further investigation, the absence of any such stimulating action by intakes within the suggested range of human requirement (32) and the absence of any leukopoietic effect from any of the doses would indicate that copper deficiency is certainly not the cause of the cytopenia.

General Discussion.—The anemia in puppies reported by Fouts

and associates (14, 15) resulting from vitamin B_6 deficiency was described as a microcytic anemia with no appreciable change in the leukocyte counts. In contrast with that, the condition we are here describing in monkeys appears to be a normocytic anemia accompanied by a profound lowering of the number of leukocytes. The two conditions therefore present distinctly different pathological pictures. Furthermore, calculated on the basis of assays reported by Birch et al. (33), our diet 600 contains approximately 28 "rat day doses" of vitamin B_6 per portion, and the modified Goldberger diet that we used contains 80 or more rat day doses of this vitamin. On the other hand, the amount of liver extract necessary to prevent nutritional cytopenia in the monkey contains only 10 rat day doses of vitamin B_6, computed from data from the same laboratory. In the light of the foregoing statements it appears quite improbable that a deficiency of vitamin B_6 could be a causative factor in the production of nutritional cytopenia in the monkey.

Since many of the monkeys ate well up to within a day or two of death, we feel justified in concluding that the cytopenia was not the result of simple inanition.

The experiments on monkeys 24, 27, and 32 demonstrate that the quantity of basal diet was adequate; all of these survived more than 500 days on the usual ration supplemented with a small quantity of an active material (yeast or liver extract), and their blood pictures remained normal. Monkey 32 is of especial interest in this connection. It received the usual amount of basal diet plus 2 gm. of Lilly's liver extract daily. On the 388th day it had reached a maximum weight of 4295 gm. Evidently the usual daily ration was not adequate to support growth in such a large animal, for during the next 288 days the animal declined in weight, although the blood picture remained normal. Consequently, the amount of basal diet was doubled on the 677th day, and by the 728th day the animal had attained a weight of 4955 gm. (Text-fig. 5). On this date the liver extract was withdrawn but the double portion of basal diet was continued. The monkey died 72 days after the withdrawal of the liver extract, having shown the typical leukopenia and gingival lesions characteristic of vitamin M deficiency. These experiments would seem to preclude the possibility that the cytopenia was the result of an inadequate caloric intake.

Monkey 27, which received 10 gm. of yeast daily, and monkey 29, which received 4 pulvules of extralin daily, developed a paralysis after they had been on the experiment for more than a year. Monkey 29 was returned to a mixed stock diet after the 572nd day, and within a short time the paralysis had disappeared;

this would suggest that a dietary factor may have been involved in the etiology of the paralysis. Monkey 32 survived for 2 years on the basal diet supplemented with 2 gm. of liver extract daily and did not show any evidence of paralysis. We have no explanation to offer for the appearance of paralysis in these 2 monkeys, but wish to record the observations in the interest of completeness.

Space will not permit a detailed comparison of these experiments on the monkey with the other experimental anemias of dietary origin described in the literature, but certain brief comments may be appropriate. One cannot avoid being impressed by the similarity of this symptom complex to the anemia and leukopenia which Rhoads and Miller have shown accompany black tongue in dogs (7, 8, 10). Also, the condition reported by Wills and associates (4, 5) in monkeys resembles in many ways the condition that we refer to as nutritional cytopenia. However, their monkeys survived longer, the blood changes appeared much later, and their animals appeared to develop a more severe anemia but milder leukopenia than our animals. Furthermore, the anemia in their animals was reported to be macrocytic, while the volume index and color index in our animals did not deviate greatly from normal. The panmyelophthisis in rats reported by György and associates (11) resembles nutritional cytopenia in the monkey in so many ways that one might be inclined to believe they had a common etiology, were it not that György has reported that nicotinic acid prevented the blood changes in their rats (12).

Although the condition we designate as nutritional cytopenia in many ways resembles certain clinical conditions, as agranulocytosis, aplastic anemia, and possibly others, it is not identical in all respects with any one of them. We feel that it is wise to defer the discussion of these points of similarity and dissimilarity until more evidence is available.

SUMMARY

Young *rhesus* monkeys (*Macaca mulatta*) were given a diet containing casein, polished rice, whole wheat, salt mixture, sodium chloride, cod liver oil, and ascorbic acid. They developed a syndrome characterized by anemia, leukopenia, and loss of weight. Ulceration of the gums and diarrhea were common, and death occurred between

the 26th and 100th day. 4 monkeys were given the deficient diet supplemented with 1 'mg. of riboflavin daily, and these developed the characteristic signs and died in periods of time similar to the survival of monkeys receiving the deficient diet alone. Nicotinic acid, either alone or in combination with riboflavin and thiamin chloride, failed to alter appreciably the course of the deficiency manifestations. Thus, it is evident that this nutritional cytopenia is not the result of a deficiency of vitamin B, riboflavin, or nicotinic acid.

The deficient diet supplemented with either 10 gm. of dried brewers' yeast or 2 gm. of liver extract (Cohn fraction G) daily supported good growth, permitted normal body development, and maintained a normal blood picture over long periods. It is obvious that yeast and liver extract contain a substance essential to the nutrition of the monkey which is not identical with any of those factors of the vitamin B complex that have been chemically identified. We have proposed the term vitamin M for this factor which prevents nutritional cytopenia in the monkey.

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EXPLANATION OF PLATE 50

FIG. 1. Photograph of mouth of monkey 26, showing necrosis of gum with exposure of maxilla. This animal received the deficient diet only. Photographed on the day of death.

FIGS. 2 and 3. Photographs of monkey 31, which received the deficient diet supplemented with 5 mg. of copper (as copper sulfate) daily. Marked ulceration and necrosis of the gums are evident. Photographed on the day of death.

THE LYMPHATIC PATHWAY FROM THE NOSE AND PHARYNX

THE ABSORPTION OF CERTAIN PROTEINS

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In a previous paper (1) the absorption, in water solution, of two colloidal dyes, trypan blue and T-1824, from the nose and pharynx was described. In this paper there are presented the results of studies on the absorption of certain solutions of proteins.

Material and Method

Experiments have been performed on 2 monkeys (*Macaca mulatta*), 12 cats, 2 dogs, and 1 rabbit. Under nembutal anesthesia a cannula was inserted into one or both cervical lymph ducts, and lymph was obtained either flowing spontaneously or, occasionally, following light massage of the neck. After samples of lymph and blood had been obtained, a solution of a foreign protein was dropped into the nose, and the collection of lymph was continued for several hours, the experiment concluding with the taking of a second blood sample. The protocol of a typical experiment may be cited in illustration.

Experiment 3.—Cat, weight 3.8 kg. Jan. 17, 1938. *9:15 a.m.*, nembutal (5 per cent) 4.0 cc. intraperitoneally. *9:50*, tracheal cannula inserted, esophagus ligated. *10:30*, left cervical lymph duct cannulated. Slight lymph flow. *11:15*, right cervical lymph duct cannulated. *1:10 p.m.*, nembutal (5 per cent) 1.0 cc. intraperitoneally. *1:12*, lymph flow now very much better, flowing on both sides on light massage of duct, on right side almost spontaneous. *2:10*, protein right cervical lymph, 4.15 per cent. *3:00*, protein left cervical lymph, 4.4 per cent. *3:42*, animal killed.

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Collection of Blood and Lymph Specimens

Time	Specimen
0:30 a.m. to 12:45 p.m....	Lymph I
	Blood I
2:45.............	1.3 cc. of egg albumin solution placed in each nostril
12:47 to 12:53...........	Lymph II
12:45 to 1:05...........	Lymph III
1:05 to 1:27............	Lymph IV
1:27 to 2:03............	Blood II
3:40..................	

Titration of Blood Serum and Lymph against Anti-Egg Albumin Rabbit Serum
(Precipitin Ring Test)

Dilution of lymph or blood serum..........	Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Lymph I (normal)..	−	−	−	−	−	−	−	−	−	−	−	−
Lymph II..........	+	+	+	+	+	+	+	+	±?	−	+	−
Lymph III..........	+	+	+	+	+	+	+	+	+	+	Slight	−
Lymph IV.........	+	+	−	−	−	−	−	−	−	−	−	−
Blood I............	−	−	−	−	−	−	−	−	−	−	−	−
Blood II..........	−	−	−	−	−	−	−	−	−	−	−	−

Protein Solutions Used. Egg Albumin.—Pure crystalline egg albumin was prepared and stored at 2°C. Shortly before use the crystals were placed in a cellophane membrane and dialyzed against distilled water until the latter no longer gave any reaction on being tested for either NH_4^+ or Cl^- ions. The solution was sterilized by passing through a Berkefeld V filter at a negative pressure of 40 cm. Hg. In most of the experiments the protein content (Kjeldahl) was 4.575 per cent. In Experiment 9, a 10 per cent solution was used. This concentration was obtained by evaporation of the protein solution contained in a closed cellophane bag suspended in air.

Horse Serum.—This was obtained from the Antitoxin and Vaccine Laboratory, Massachusetts State Department of Public Health.

Serum Albumin.—This was purified by crystallization and stored at 2°C., with the addition of a small amount of toluol. Before use it was treated in the same way as the egg albumin.

Horse Serum Plus T-1824.—For Experiment 12, there was used a 5 per cent solution of T-1824 in horse serum; for Experiment 13, a 1 per cent solution.

Protein Detection.—Normal rabbits, weighing about 2 kg., were injected from 2 to 4 times (intravenously) with the individual protein solutions until their sera exhibited a good specific precipitin titre. Blood was then withdrawn from the

heart and the sterile serum stored at 2°C.   Not infrequently non-specific (cross) precipitin reactions were encountered.

Samples of normal lymph and blood serum were collected at the start of each experiment.   These served as control for any non-specific precipitin reactions. No cross reactions occurred in any of the egg albumin experiments, but they were most frequent where horse serum was the protein and cats the experimental animals.

RESULTS

Egg Albumin.—With the exception of one experiment egg albumin was always found in the lymph after a short interval (Table I).

Horse Serum.—Horse serum dropped into the nose was never detected in either lymph or blood.

Horse Serum Plus T-1824.—Gregersen and Gibson (2) suggested that T-1824 in the blood combined in some way with the plasma proteins.   Since T-1824 in aqueous solution passes readily through the nasal mucosa (1), whereas horse serum does not, it seemed reasonable to suppose that by using a solution of T-1824 in horse serum this hypothesis could be put to the test, using the nasal mucosa as a biological dialyzing membrane.   When employing a 5 per cent solution of T-1824 in horse serum, a small amount of dye appeared in the lymph, though very much less than when using a solution of equal strength in normal saline.   With a 1 per cent solution of T-1824 in horse serum no dye whatever appeared in the lymph, whereas with a solution of the same strength in saline the dye came through quite freely.   These observations therefore appear to support the view of Gregersen and Gibson (2) that T-1824 combines with the blood proteins, but suggest also that in the case of the 5 per cent solution there was a slight excess of dye which did not combine, and that it was only this excess which passed through the nasal mucosa.   In the 1 per cent solution, on the other hand, practically all the dye was combined with the serum and consequently none came through.

Serum Albumin.—A solution of a pure serum albumin was furnished by the Department of Physical Chemistry of The Harvard Medical School.   This was never found in either lymph or blood in the cat, but was found in the rabbit lymph.

Table I presents in a summarized form the essential data of all our experiments.

TABLE I

Summary of All Experiments in Which Protein Solutions Were Placed in the Nose and Their Presence Then Tested for in Cervical Lymph

Experiment No.	Animal	Protein	Amount placed in nostrils		Time placed in nostrils	Time noted in lymph	Remarks
			Right	Left			
			cc.	cc.			
1	Cat	Egg albumin	1.5	1.5	12:57 to 1:10	1:20	
2	"	"	1.5	1.5	1:10 to 1:20	1:23	
3	"	"	1.3	1.3	12:47 to 12:53	1:16	
4	"	"	1.2	1.2	11:54 to 12:00	12:38	Cervical ducts not cannulated. Experiment to see whether albumin would be found in blood if lymphatics undisturbed (see text)
5	"	"	1.5	1.5	12:25 to 12:35	12:45	
6	"	"	1.5	1.5	12:33 to 12:38	Not found	
7	Dog	"	1.5	1.5	11:40 to 11:42	1:33	
8	"	"	3.0	3.0	12:25 to 12:29	1:00	
9	Monkey	"	3.0	None	12:15 to 12:17	Not found	
10	Cat	"	1.5	1.5	11:48 to 11:56	"	
11	Monkey	Horse serum	1.5	1.5	1:15 to 2:40	"	
12	Cat	"	1.5	1.5	12:52 to 12:58	"	
13		Horse serum plus T-1824 (5 per cent)	1.5	1.5	1:38 to 1:42	1:35	Small amount of T-1824 came through (see text)
14		" " (1 per cent)	1.5	1.5	12:55 to 1:10	Not found	No T-1824 came through (see text)
15		" "	1.5	1.5	12:00 to 12:07	"	
16		Serum albumin	1.5	1.5	11:25 to 11:30	"	
17	Rabbit	"	1.5	1.5	12:30 to 12:40	1:50	

DISCUSSION

The cavities of the nose and pharynx are in direct continuity with the remainder of the alimentary canal. In air-breathing vertebrates, however, the nose and pharynx have undergone a functional specialization. Under normal conditions the nasal mucosa no longer comes in contact with ingested foods, while the pharynx does so for only a brief period, during deglutition. Nevertheless the nasal mucous membrane still possesses some power of absorption. This has been demonstrated by Blumgart for pituitary extract (3), lead carbonate (4), and potassium ferrocyanide (5), by Cohen, Ecker, and Rudolph (6) for ragweed pollen, and by Van Dellen, Bruger, and Wright (7) for mecholyl. Several observers have also noted the presence of dye in the cervical lymph nodes after nasal instillation (Clark, 8; Farber, 9). The present findings constitute the first direct observation of the absorption of unsplit protein from the nose and pharynx. Whether any proteolysis occurs in the nasopharynx has not been investigated.

Is there direct absorption into the blood? With the colloidal dyes T-1824 and trypan blue it has been shown (1) that absorption takes place not only *via* the lymph but also directly into the blood stream. If no cervical lymph is permitted to enter the blood, the blood is nevertheless found to contain the dye in increasing concentrations. In none of our experiments, however, has it been found possible to detect egg albumin in the blood under any conditions—not even in Experiment 5, where the egg albumin solution was placed in the nose and the cervical ducts left intact. This is probably an expression of the small amount of lymph entering the blood.

Apart from the nose and pharynx, a great deal of work has been done on the absorption of unsplit protein from the remainder of the alimentary canal. Ratner and Gruehl (10) give an excellent review of the literature. That such absorption occurs has been concluded by other workers (*e.g.*, Walzer and Walzer, 11) who have been able to induce hypersensitiveness to a given protein after oral administration. Banks (12) was unable to find any evidence of this protein being absorbed *via* thoracic duct lymph. Alexander, Shirley, and Allen (13), also using the precipitin ring test, detected egg albumin in dogs—after feeding by stomach tube—in thoracic duct lymph but not in portal blood.

Relation between Protein Absorption and Molecular Weight.—Egg albumin is the protein most readily and constantly absorbed. Among the proteins commonly encountered it has the lowest molecular weight.

Proteins used	Molecular weight
Egg albumin....................	34,500 (Svedberg and Nichols, 14)
Serum albumin....................	72,000 ± 3,000
Serum globulin....................	175,000 (Adair and Robinson, 15)
Horse serum....................	Mixture of serum albumin and globulin

Region from Which Absorption Occurs.—Blumgart (4) states that absorption occurs only from the olfactory area proper. We have as yet no evidence bearing on this point.

The trachea was cannulated and the esophagus ligated as routine procedures in order to eliminate the possibility of protein being absorbed into the blood from the lungs or the gastrointestinal tract. This raises the obvious objection that our experimental conditions were too artificial, that even if protein was absorbed from the pool thus held up in the nose and pharynx, it might not have been absorbed had the trachea and esophagus been left open. However, in a control experiment (Experiment 6) in which the trachea and esophagus were left intact, egg albumin appeared in the cervical lymph 12 minutes after it had been dropped into the nose. This must mean that the actual passage through the nasal mucosa takes place very rapidly.

No Deterioration of the Nasal Mucosa.—An obvious question is whether our experiments deal with a normal nasal mucosa. It is possible that in the course of an experiment lasting several hours there may be a progressive deterioration in the condition of the mucous membrane. It must be remembered, however, that dye (1) and protein (see previous paragraph) pass through the mucosa within a few minutes after being placed in the nose. At this period the mucous membrane has had no time to deteriorate and must be as near normal as it can possibly be. No volatile anesthetics were used. There is no question of the mucosa being damaged by irritant vapors.

SUMMARY

1. In a number of cats, dogs, monkeys, and in a rabbit, the cervical lymph ducts were cannulated and protein solutions dropped into the

nose, and the lymph was examined afterwards for the presence of the protein employed.

2. Egg albumin was found in the lymph in all cases with one exception.   Horse serum was never detected.   Serum albumin did not come through in the cats, but did in a rabbit.

3. With a 1 per cent solution of T-1824 in horse serum no dye appeared in the lymph.   This is regarded as confirming Gregersen and Gibson's (2) view that T-1824 combines with the serum proteins.

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INDEX TO AUTHORS

INDEX TO SUBJECTS